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Examining the Behavioral and Molecular Aspects of Adolescent Nicotine Dependence: Implications for Vulnerability to Drugs of Abuse

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EXAMINING THE BEHAVIORAL AND MOLECULAR ASPECTS OF ADOLESCENT NICOTINE DEPENDENCE: IMPLICATIONS FOR VULNERABILITY TO DRUG ABUSE

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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Dedication

I would like to dedicate this work to my grandparents, Bill and Dee Monroe. To my grandfather; he has shown me the importance of education, family, and upholding yourself to the highest level of standards. And to my grandmother whose love and encouragement is unfailing. Thank you for taking great interest in what I am studying and always keeping me up to date with the latest news in smoking cessation!

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LIST OF ABBREVIATIONS

5HT	serotonin
AUC	area under the curve
Ca^{++}	
CAMK	Ca^{2+}/Calmodulin kinase
CL	confidence limits
CPP	conditioned place preference
CRE	cyclic AMP response element
CREB	cyclic AMP response element binding protein
DA	dopamine
DHBE	dihydro-beta-erythroidine
DRC	dose response curve
ED_{50}	effective dose 50%
EPM	elevated plus maze
ERK	extracellular regulated kinase
FR	fixed ratio
GABA	gamma-aminobutyric acid
HIP	hippocampus
i.p.	intraperitoneal
i.v.	intravenous
Inj	injection
MCL	mesocorticolimbic reward pathway
mg/kg	milligrams/kilogram
min	minutes
MP	mini-pump
MPE	maximal percent effect
NAC	nucleus accumbens
nAChR	nicotinic acetylcholine receptor
PFC	prefrontal cortex
PKA	protein kinase A
PND	postnatal day
s.c.	subcutaneous
SE	standard error
sec	seconds
SEM	standard error of the mean
STR	striatum
TH	tyrosine hydroxylase
VTA	ventral tegmental area

Abstract

EXAMINING THE BEHAVIORAL AND MOLECULAR ASPECTS OF ADOLESCENT NICOTINE DEPENDENCE: IMPLICATIONS FOR VULNERABILITY TO DRUG ABUSE

By Dena Heath Kota, B.S.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Approximately 200 million men and 100 million women smoke worldwide. In the United States, an estimated 25.9 million men (23.9 percent) and 20.7 million women (18.1 percent) are smokers. The commencement of smoking at a young age is thought to increase addiction liability, decrease the probability of successful cessation, and correlate with a higher number of cigarettes smoked per day. Studies from the World Health Organization indicate that between 80,000 and 100,000 children start smoking every day worldwide. These statistics suggest that adolescence is a critical phase for developing nicotine dependence. The work in this dissertation contributes to the further

understanding of this unique developmental period. Our research shows that various aspects of nicotine dependence are both age- and sex-dependent. We observed age- and sex-related differences in both nicotine reward and withdrawal models that imply a heightened vulnerability for adolescents. In addition, we have investigated possible behavioral and molecular mechanisms which may underlie the elevated vulnerability to dependence. The data illustrate that while behavioral mechanisms only play a minor role in the differences seen in reward and withdrawal, molecular mechanisms appear to have a greater contribution. Specifically, increased nicotinic receptor function is likely to be a substantial contributor to age-related disparities. In addition, nicotine is one of the first and most commonly abused drugs in adolescence and is known to be a strong predictor of subsequent alcohol and other drug abuse. Our research investigated the effects of adolescent nicotine exposure on both nicotine and cocaine dependence in adulthood. We found that exposure to nicotine during the early phase of adolescence affects both nicotine reward and withdrawal in adulthood. Moreover, this exposure also bears impact on other drugs of abuse such as cocaine. In summary, our data suggest that early adolescence is the most critical period for becoming dependent to nicotine and that early experimentation with nicotine may lead to enhanced vulnerability to dependence on more illicit drugs of abuse. It is imperative that we understand why adolescents have a heightened susceptibility to nicotine dependence so that better smoking cessation therapies and prevention messages can be developed for this age group.

GENERAL INTRODUCTION

A. Tobacco Smoking and Nicotine Dependence

In the United States, an estimated 25.9 million men (23.9 percent) and 20.7 million women (18.1 percent) are smokers (National Health Interview Survey (NHIS), 2005, National Center for Health Statistics). More importantly, smoking related-diseases kill one in ten adults globally, or cause four million deaths. By 2030, if current trends continue, smoking will kill one in six people (World Health Organization Smoking Statistics 2002). Nicotine addiction is not only a problem for the adult population. Surprisingly, over 6,000 teenagers begin smoking every day (American Lung Association Statistics 2002). Moreover, 90% of adult smokers report their first use of tobacco prior to age 18 (Chassin et al. 1990). Tobacco is reportedly the most avoidable cause of disease and disability, yet less than 7% of smokers who attempt to quit actually achieve more than one year of abstinence before they relapse (NIH Pub. No. 98-4342, CDC Prevention and Health Promotion, 2005). Smoking is a significant and preventable health concern that needs further attention so that sophisticated health promotion campaigns and messages can be imparted to the public.

Nicotine is the primary addictive component in tobacco that acts on the brain to produce both rewarding and aversive effects (Castane et al. 2005). Although nicotine is known to reach the brain rapidly, it does not have long lasting acute effects due to its short half-life of 1-2 hours (Viveros et al. 2006). This property of nicotine is likely to contribute to its repeated and consistent use. In addition, environmental cues play an

important role in smoking addiction as well. Several human and rodent studies have investigated the importance of cravings and contextual cues in smoking behavior using conditioned place preference models. One study found that environmental cues related to smoking activate certain CREB-related molecular pathways in the brain; therefore eliciting the same effects as direct exposure to nicotine (Walters et al. 2005). Another study examined brain activity in regions associated with attention, motivation, and reward while participants viewed a series of pictures of smoking-related objects and scenes (McClernon et al. 2005). Study participants provided self-reports of cravings before, during, and after each session. Researchers found that smokers who reported a greater urge to smoke following the period of abstinence also exhibited stronger brain activity after viewing smoking-related images. In contrast, smokers who reported fewer cravings displayed stable or decreased brain activity, despite viewing the same smoking-related images after a period of abstinence (McClernon et al. 2005). These differences may influence levels of cigarette craving following abstinence and may also affect the impact of smoking cues. Smokers who experience a greater sensitivity to smoking cues may have difficulty quitting smoking and may also be more prone to relapse.

B. Adolescence and Smoking

Tobacco smoking at a young age is an increasing problem in the United States and around the world. The rate of adolescent smoking among Americans has been rising sharply since 1992 (Johnston et al. 1998). Moreover, the age of initiation for smoking has also been declining (Johnston et al. 1998). The commencement of

smoking at a young age is thought to increase addiction liability, decrease the probability of successful cessation (Colby et al. 2000; Kandel and Chen 2000), and correlate with a higher number of cigarettes smoked per day (Taoli and Wynder 1991). Studies from the World Health Organization provide evidence that around 50% of those who start smoking in adolescence go on to smoke for 15 to 20 years (2003). These statistics should indicate the critical nature of providing influential prevention messages at an early age. The longer a child or teenager is prevented from smoking, the higher the chance of preventing lifetime dependence.

Despite the fact that initial exposure to nicotine has been shown to be unpleasant (Eissenberg and Balster 2000), many adolescents still go on to become dependent on this drug despite the desire to quit. Even though adolescent tobacco intake is thought to be lower than that of adults, this age group also experiences signs of withdrawal such as cravings, nervousness, and the inability to concentrate (Rojas et al. 1998; Killen et al. 2001). In fact, adolescent smokers report frequent unsuccessful attempts to quit due to cravings and withdrawal symptoms (Johnson 1982; Biglan and Lichtenstein 1984). Certainly many factors are involved in an adolescent's decision to maintain a regular level of smoking. These include, but are not limited to, social pressure, environment, stress, biological effects, reinforcing effects, and aversive withdrawal symptoms. These studies show that further investigation is needed regarding adolescent nicotine dependence and cessation therapies. Indeed, Colby et al. (2000) wrote a review suggesting that the current methods and approaches to smoking cessation in adolescence need additional attention since successful cessation rates are

modest. It is critical that we understand why this age group is particularly vulnerable to nicotine dependence and addiction so that better prevention messages and smoking cessation therapies can be developed.

The Transition of Adolescence

Adolescence is a critical decade of transition that occurs between a fully dependent child and gaining independence as an adult. During this period, many changes occur in a variety of areas such as physical growth, cognition, social skills, physiology, and emotions. Since this developmental stage induces alterations of a number of biological systems at one time, it is natural to assume that the adolescent will experience an increased vulnerability to a wide range of biological and behavioral problems. One of these issues is that of substance abuse. It has been shown that the adolescent brain may be more susceptible to the effects of addictive substances such as alcohol, nicotine, and cannabis among others (Spear 2000; Smith 2003; DiFranza 2007). Furthermore, studies have also demonstrated that early use of any drug is a strong indicator of regular drug use in adulthood (Toumbourou et al. 2005; Teeson et al. 2006). These studies impart the importance of effectively framing prevention messages, developing prevention strategies, and implementing useful public education tactics before adolescents begin drug experimentation.

Adolescent Brain Development

The unique timing of adolescent brain development is thought to be a large contributor to the heightened vulnerability to substance abuse. While the brain of a young child is almost 95% of the size of an adult brain, there are many neuroanatomical

differences that yield dissimilar abilities to think and reason. In general, studies have shown that the adolescent brain develops from back to front and from bottom to top; thus the areas associated with emotion, instinct, and pleasure develop first. These areas include the amygdala, ventral tegmental area (VTA), and nucleus accumbens (NAc). Last to develop is the prefrontal cortex (PFC) which is responsible for critical thinking and judgment. This imbalance leads to the activities which are often associated with the hallmarks of adolescent behavior; that they tend to be impulsive and emotionally driven, lack self-control and planned thinking, and demonstrate increased risk-taking behavior. It is suggested that this maturation pattern contributes to an increased propensity toward substance abuse at a young age.

Each brain region is susceptible to an immense amount of remodeling and maturation. The PFC, in particular, undergoes many modifications during the adolescent period. Volume of this region decreases in humans (Sowell et al. 1999) and rats (van Eden et al. 1990). Furthermore, density of spines on pyramidal cells in the human PFC decline (Mrzljak et al. 1990). On the other hand, dopaminergic (DA) input to the PFC peaks during this phase (Lewis 1997; Brenhouse et al. 2008), as does the quantity of DA transporters (Akbari et al. 1992). In addition to these increases in dopaminergic input, an increase in the number of DA receptors has also been reported (Seeman et al. 1987). While transformations of neural circuitry are not limited to the DA system, these changes are thought to play a critical role in the rewarding and reinforcing effects of many drugs of abuse, including nicotine.

In general, the adolescent brain goes through a vast amount of pruning, synapse loss, and alterations of neurobiological pathways. It has been estimated that as many of half of the average number of synapses are lost during adolescence (Rakic et al. 1994). The function of this synaptic loss is not yet fully understood, but is assumed to have a developmental purpose. One which has been suggested by Rakic et al. is that since many of the synapses in adolescence are excitatory, the pruning serves to decrease unnecessary excitatory stimuli to the brain. In addition, a variety of receptors (DA, serotonin, GABA, acetylcholine) tend to be overproduced and subsequently pruned during this period. (Lidow et al. 1991; Lidow and Rakic 1992). Collectively, adolescence is a period of intense neurological development and many of the changes which are ongoing during this period may play a role in subsequent drug abuse.

The Role of Gender in Nicotine Dependence

Gender and sex differences in response to the behavioral effects of drugs have long been reported in humans and rodents (Bradley et el. 1968; Camp and Robinson 1988; Sircar and Kim 1999; Damaj 2001; Hughes 2006; Jones et al. 2006). However, most of the published work is related to the adult. Indeed, female mice demonstrated lower sensitivity to the acute effects of nicotine when tested in an acute thermal pain model (Damaj 2001). Decreased nicotine sensitivity in females has also been shown in several human studies (Jamner et al. 1998; Perkins et al. 1999). In reward paradigms, adult female rats displayed a shorter latency to the first nicotine infusion in a self-administration model and demonstrated faster acquisition of nicotine self-administration behavior (Donny et al. 2000; Chaudhri et al. 2005). Pharmacokinetic and distribution

factors seem to play an important role in explaining these differences. Indeed, Benowitz showed that women exhibit faster nicotine and cotinine metabolism as compared to men (2006), possibly due to hormonal influences. This observation suggests that females may smoke more cigarettes to obtain desired levels of nicotine in the body resulting in a greater exposure to tobacco toxins and increased levels of addiction. Neurobiological differences appear to contribute significantly to these sex differences as well. Sex is a factor that influences many areas of the brain including (but not limited to) memory, emotion, pain perception, neurotransmitter signaling, and stress hormones. Structural and functional differences have been shown in the hippocampus (Juraska 1991), amygdala (Hines et al. 1992), striatum and nucleus accumbens (Becker 1999). Furthermore, there are unique differences in serotonin (Carlsson and Carlsson 1988) and dopamine (Becker 1999) transmission. These dimorphisms in signaling could lead to significant differences in behavioral responses, particularly those involving addiction pathways.

Limited work on sex and gender differences in adolescent smoking has been done. Even though adolescents have yet to reach full sexual maturity, some studies do suggest differences in smoking behavior and nicotine sensitivity. Levin et al. (2003) has shown that female rats which initiate nicotine i.v. self administration in young adulthood exhibit higher nicotine intake as adults relative to rats that initiate self administration in adulthood. Additionally, adolescent female mice have been shown to voluntarily consume nicotine orally in a dose-dependent manner and to consume significantly more than male adolescents (Klein et al. 2004). As with adults, social and

environmental factors are likely to contribute to progression from use to addiction in adolescents, but there is undoubtedly a biological basis as well. Given that nicotine use often begins in adolescent years, it is reasonable to speculate that developmental changes are important contributors. Our studies aim to thoroughly investigate the biological and pharmacological factors that are critical to nicotine abuse. It is important to begin with the basic CNS physiological and pharmacological effects of nicotine in order to fully understand the complexities of its abuse liability.

C. Molecular and Pharmacological Mechanisms Involved in Nicotine Dependence

Nicotinic Receptors

It is clear that nicotine has many central and peripheral effects which potentially contribute to its addictive properties. Acute administration of nicotine elicits various central responses including antinociception, hypoactivity, and hypothermia. Nicotine is also known to have effects on other body systems including the cardiovascular (CV) and gastrointestinal (GI) systems. Use of nicotine causes increases in heart rate and blood pressure which is a concern for already hypertensive smokers (Benowitz 2003). In the GI tract, nicotine is a known to cause smooth muscle relaxation via release of nitric oxide (Irie et al. 1991). Nicotine exerts these physiological effects by binding to nicotinic acetylcholine receptors (nAChRs) in the brain and the periphery. More chronic nicotine exposure can lead to physiological dependence as a result of both the rewarding and aversive properties induced by the drug.

Nicotinic acetylcholine receptors can be found in many locations throughout the body. Neuronal receptors are found in the central nervous system (CNS) and the

peripheral nervous system (PNS), while neuromuscular receptors are found in neuromuscular junctions. These ionotropic receptors are ligand gated ion channels which are pentameric in structure meaning they are comprised of five subunits arranged around a central pore. This central pore allows for the passage of cations such as sodium, potassium, and calcium. Receptors can be a homomeric or heteromeric composition of different subunits. The neuromuscular nAChR is composed of two α subunits, one β, one δ, and either a γ or ε subunit, while the neuronal nAChR can consistent of subunits ranging from α2- α10 and β2- β4 making them much more heterogeneous. With such a wide variety of receptor subtypes, many different pharmacological effects can occur upon nicotine administration.

The binding of a nicotinic agonist to the receptor causes a change in the conformational state which leads to activation. That is, an agonist causes the gated ion channel to open rapidly (activation) and then become inactive for a period of time (desensitization) before returning to a resting state which is inactive, yet capable of reactivation. Ligand-bound desensitization of the nicotinic acetylcholine receptor was first characterized by Katz and Thesleff (Pitchford et al. 1992). This phenomenon is often caused by prolonged or repeated exposure to a drug and results in decreased responsiveness of that receptor to a stimulus. It can further lead to upregulation of nAChRs in order to compensate for the lack of response to nicotine. This compensatory mechanism is suggested to contribute to nicotine dependence and addiction (Changeux et al. 1998; Buisson and Betrand 2002; Nashmi and Lester 2007).

Many of nicotine's pharmacological effects are the result of activation of a variety of nAChR subtypes. Although the precise mechanisms of these effects have not been elucidated, recent research using genetic knockout models and pharmacological ligands has contributed to further understanding. The majority of nAChRs in the CNS contain either $\alpha4\beta2^*$ heteromers or α7 homomers (Changeux et al. 1998). While it is known that five α7 subunits compose the homomeric receptor, the α4β2* heteromers can be composed of a variety of additional subunits including α5, α6, or β3 which lead to diversity in receptor characteristics. Several studies have implicated the α4β2* subtype, which is present throughout the mesolimbic dopamine pathway, in the reinforcing effects of nicotine. Heteromeric α4β2* nAChRs are localized on dopaminergic and GABAergic neurons in the VTA. Stimulation of dopaminergic receptors often results in enhanced dopamine release, whereas the desensitization of nAChRs on GABA neurons is thought to attenuate the GABA-mediated inhibitory drive (Mansvelder et al. 2002; Cohen et al. 2005; Solinas et al. 2007). In addition, nicotine interacts with nAChRs on glutamate neurons that regulate the activity of DA and GABA neurons in the VTA. Taken together, this pattern of brain pathway activation is likely to result in the enhanced rewarding and reinforcing effects which contribute to nicotine addiction.

Indeed, preclinical studies in transgenic mice have shown that elimination of either the α4 or β2 subunit attenuates the pharmacological and behavioral effects of

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[*] minor populations of α4β2 nAChRs may contain additional unknown subunits as defined by the asterisk (Lukas et al. 1999)

nicotine (Picciotto et al. 1998; Marubio et al. 1999). Specifically, the β2 subunit has been shown to be necessary for nicotine-induced conditioned place preference in mice (Walters et al. 2006). In addition, targeted expression of β2 subunits in the VTA of β2-knockout mice reinstates nicotine-induced DA release (Maskos et al. 2005). Data has confirmed a role for this subtype in nicotinic withdrawal as well. Pharmacological nicotine withdrawal studies demonstrated that utilizing the β2-selective antagonist, dihydro-β-erythroidine (DHβE), resulted in anxiety-related behavior and elevations in reward threshold, which are measures of affective signs of nicotine withdrawal (Damaj et al. 2003; Bruijnzeel et al. 2004; Jackson et al. 2008).

Molecular Pathways Involved in Drug Dependence

One of the main goals in the field of nicotine research is to gain a better understanding of the mechanisms which underlie nicotine dependence in order to advance current smoking cessation therapies and prevention programs and reduce the number of smoking-related illnesses and deaths. Even though many drugs exhibit differing acute actions, the majority of drugs of abuse converge on similar reward circuitry in the brain that has been shown to be involved in addiction and dependence. The brain contains a specialized pathway, often referred to as the mesocorticolimbic (MCL) reward pathway, which has been implicated in many of the rewarding and reinforcing effects of drugs of abuse (Nestler 2001; Kobb and Le Moal 2001). This pathway originates in the ventral tegmental area (VTA), near the base of the brain. Neurons from this region send projections to target regions in the front of the brain, most notably to the nucleus accumbens (NAc) (Nestler 2001; Hyman and Malenka

2001). Indeed, this circuit is a critical component of reward physiology in that animals with lesions in these regions exhibit a loss of drug consumption (Robinson and Berridge 2001; Nestler 2004). Dopamine is the most common and essential neurotransmitter involved in this pathway. Nicotine, in particular, is able to activate VTA dopaminergic neurons directly via stimulation of nicotinic cholinergic receptors or indirectly via stimulation of its receptors on glutamatergic neurons which then innervate dopamine cells. In addition to nicotine, cocaine is also able to elevate dopamine production in another manner. This drug, as well as other psychostimulants, inhibits the return of dopamine to the VTA by blocking dopaminergic transporters; thus resulting in an accumulation of dopamine in the junction (Nestler 2001).

The stimulation of nAChRs by pharmacological agents can lead to many downstream consequences. One of the most prominent and readily released second messengers following nAChR activation is calcium. Calcium can act downstream on a number of targets including Ca^{2+}/calmodulin-dependent protein kinases (CaM kinases) and protein kinase A (PKA). This in turn leads to stimulation of the extracellular signal-related kinase (ERK) pathway and activation of cAMP response element binding protein (CREB) which has been implicated in drug addiction (Brunzell et al. 2003). The transcription factor CREB is thought to play a major role in the rewarding properties of many drugs of abuse. In particular, Walters et al. (2005) has shown that activation of CREB is necessary for nicotine reward in adult mice as measured by conditioned place preference testing. The involvement of CREB in nicotine withdrawal remains more complicated. Chronic nicotine administration in mice results in decreased

CREB phosphorylation in the NAc but increased CREB phosphorylation in the prefrontal cortex, while nicotine withdrawal increased CREB phosphorylation in the VTA (Brunzell et al. 2003). In contrast, withdrawal from chronic nicotine in rats decreased CREB, phosphorylated CREB, and CRE-DNA binding in the cortex and amygdala (Pandey et al. 2001). The involvement of CREB in reward and withdrawal pathways has been studied due to its connection with the formation of dopamine. CREB can activate the enzyme, tyrosine hydroxylase (TH), which is required for dopamine synthesis. When more dopamine is produced, reward is thought to increase so the molecular players in this pathway are often examined when investigating drug dependence. (See Figure 1 for schematic of pathway). We have focused on this pathway since it remains the main mechanistic pathway which has been implicated regarding nicotine addiction. Other pathways are still possible, but have not been well-characterized to date. We have chosen to begin our studies with the nAChRs since they are the initial target of nicotine.

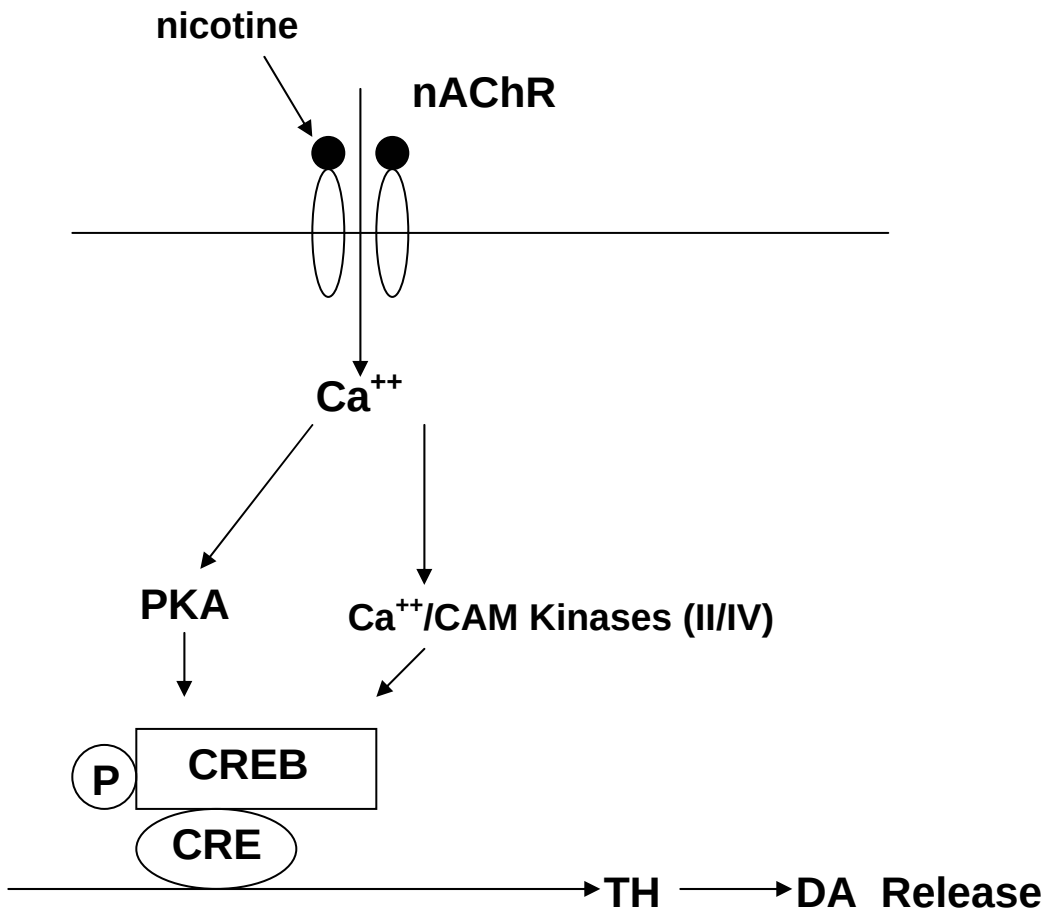

Figure 1. Possible schematic pathway for the effects of nicotine.

D. Behavioral Models of Nicotine Dependence

Animal models allow researchers to investigate basic neurochemical mechanisms of drug abuse, factors involved in drug dependence, and potential treatment for these problems. Several behavioral models have been established in order to consistently investigate the role of biological factors involved in nicotine dependence. They have also been established due to the prohibitive factors often associated with clinical studies such as cost, ethical concerns, and retaining an appropriate number of subjects for follow-up visits. Four critical components of nicotine dependence have been featured in animal models and are investigated in our studies.

Reward and Reinforcement

Drugs of abuse elicit pleasurable effects which often contribute to their repeated use and abuse. Rewarding properties of nicotine are most commonly assessed through either self-administration models or conditioned place preference (CPP) models. Conditioned place preference is a method which has been used extensively to assess the acute rewarding effects of a drug by pairing it with a particular context (Bardo et al. 1995; Tzschentke 1998). Unlike other models, such as self-administration, this model does not directly measure drug reinforcement; rather it is a measure of a preference for a context which is associated with the drug stimulus. CPP models allow researchers to assess rewarding effects of drugs without facing the technical challenges of establishing self-administration in the mouse. Moreover, as demonstrated throughout the literature, there is a reasonable concordance between drugs that produce a CPP and drugs that are

self-administered (Bardo and Bevins 2000) and data from this method serves to compliment self-administration data. This model is well-established and several reports have concluded that nicotine is able to induce CPP in rodents over a wide range of doses and with various routes of administration (Le Foll and Goldberg 2005; Grabus et al. 2006; Walters et al. 2006). This approach is advantageous because it has a short duration, does not stress the animal with surgery or extensive training and tests the animal in a drug-free state.

Nicotine Withdrawal

Rodent models have also been used to study nicotine withdrawal in humans since this is a common reason given for relapse after smoking cessation (Piasecki et al. 2000). Nicotine infusion is accomplished by various routes of administration such as osmotic mini-pumps, repeated injections, i.v. infusions, or through drinking water. Withdrawal may be precipitated by pharmacological antagonists or spontaneous removal of mini-pumps, promoting physical somatic signs (i.e. tremors, head shakes, excessive grooming and ptsosis) and negative affective signs (i.e. anxiety, irritability, and depressed mood), which are important determinants in nicotine dependence (Hughes et al. 1991; Markou et al. 1998; Damaj et al. 2003; Cohen et al. 2005; Viveros et al. 2006).

Acute Sensitivity

Nicotine exerts many pharmacological effects in the peripheral and central nervous systems following acute administration. Initial sensitivity models are useful in that they provide information on the immediate response to a drug. These initial effects

can provide insight into the biological systems on which the drug will act as well as any individual differences that may be useful in identifying at-risk individuals before they become addicted to certain drugs.

Our lab utilizes a well-characterized battery of tests which assess centrally-mediated nicotinic effects. This battery can provide information concerning nicotine potency, drug time-course, site of action, and receptor subtypes activated. Testing includes two measures of antinociception, change in body temperature, and a measure of locomotor activity. Commonly, nicotine induces increased antinociception, hypothermia, and hypolocomotion in the mouse. Antinociception, or the lack of pain response, is measured using both the tail flick and hot plate tests which are mediated by spinal and supraspinal reflexes respectively.

Tolerance

Tolerance can be defined as the capacity of the body to become less responsive to a particular substance; usually after chronic exposure to that substance. This aspect of dependence is well-established with nicotine in rodents and humans (Stolerman et al. 1974; Marks et al. 1983; Perkins et al. 1994). In effect, if a person is tolerant to a drug, such as nicotine, he will require more of that substance in order to achieve satisfactory levels of reward or other pleasurable effects. This often leads to increased drug use and can further levels of drug dependence. As with the withdrawal model, rodent tolerance commonly uses mini-pumps to administer nicotine subcutaneously. Animals are then challenged with various doses of nicotine to examine antinociceptive and hypothermic

effects. Tolerance models provide insight into which drugs are more likely to be commonly abused due to reduced physiological effects after repeated exposure.

E. Animal Models of Adolescence

The period of adolescence offers a particular challenge when developing useful animal models. A great deal of effort has gone into adapting appropriate adult animal models to those which accurately reflect adolescent development. Adolescent animal models, which have face (whether a test appears to be a good measure) and predictive (degree to which inferences can legitimately be made) validity, have been designed in order to better understand the biological factors which are involved in nicotine addiction at this age. Limitations of these models often include deciding appropriate age correlations and divisions as well as determining neurobiological correlations. Adolescent models must also consider differing drug intake patterns of adolescents, the influence of a lack of sexual maturity and assessing proper dosing regimens due to pharmacokinetic differences in rodents.

Three developmental phases of rodent adolescence have been identified: early- (PND 21-34), mid- (PND 35-46) and late- (PND 47-59) adolescence (Spear 2000; Laviola 2003). These classifications are based on similarities in physical, social, and biological development of both rodents and humans. While there are some species differences and these classifications can vary slightly, these divisions have been carefully researched and are considered the standard for research on the adolescent period.

Behaviorally, many characteristics of the adolescent period are universal across species. These traits are thought to bear adaptive and evolutionary value for all species and will allow adolescents to properly transition into adulthood with all the required skills. For example, much like human adolescents, adolescent rodents exhibit an increase in the amount of social interaction time and a peak in play behavior (Primus and Kellogg 1989). Another hallmark characteristic of adolescence is increased risk taking. Indeed, over half of adolescents exhibit risk taking behaviors such as drunk driving, sex without contraceptives, and use of illegal drugs (Arnett 1992). Similarly, adolescent mice have been noted to exhibit hyperactive behavior in a novel environment (Darmani et al. 1996) and a higher degree of novelty seeking as compared to adults (Adriani et al. 1998).

In addition to behavioral consistencies, neural alterations in humans and rodents appear to have correlations. There is a high degree of PFC remodeling noted in both human adolescents (Jernigan et al. 1991) and in rats (van Eden et al. 1990). Moreover, there are notable increases in dopaminergic input to the PFC (Kalsbeek et al. 1988) and DA transporters (Akbari et al. 1992). These parallels in behavioral and neural development have allowed researchers to develop and refine animal models of human adolescence that allow for investigation of this critical period.

An important limitation of using animal models for adolescent research is that the intake behavior of human adolescents is often different from that observed in the adult. It is difficult to mimic a sporadic and unpredictable pattern of human adolescent smoking behavior in an animal model which may limit validity of the model to some

degree. It is also important to consider pharmacokinetic differences between adults and adolescents. Adolescents are known to have increased metabolism as compared to adults (Trauth et al. 2000) which may require increasing the dose of nicotine so that metabolite levels are consistent when testing is being performed and when comparing data to that of adults.

F. Effects of Adolescent Nicotine Exposure

It is common for adolescents to first experiment with easily accessible drugs such as alcohol and tobacco. Furthermore, it is also common for this type of drug use to lead to the use of more illicit drugs of abuse. Indeed, nicotine is one of the first and most commonly abused drugs in adolescence and is known to be a strong predictor of subsequent alcohol and other drug abuse (Kandel et al. 1992). Several studies have demonstrated that a minimal amount of smoking at a young age can lead to nicotine addiction and dependence (Benowitz et al. 1994; DiFranza 2007).

Nicotine exposure during adolescence may have detrimental effects since this is a period of high neuronal plasticity and brain development. Indeed, the level of nAChRs can increase in the brain the day after the first exposure to nicotine (Abreu-Villaca et al. 2003) implying a very rapid timeline for brain remodeling following drug exposure. Furthermore, this nAChR upregulation persists at significant levels one month after treatment (Abreu-Villaca et al. 2003). Moreover, animal studies have demonstrated that adolescent nicotine exposure can cause alterations in the cholinergic, serotonergic, dopaminergic, and noradrenergic systems (Kelley and Middaugh 1999; Trauth et al. 1999) which all have roles in the reinforcing effects of other drugs of

abuse. Among these effects are neuronal cell death and changes in nicotinic receptor expression (Trauth et al. 2000). Perhaps most important, the behavioral responses of other drugs of abuse are known to be altered by adolescent nicotine exposure suggesting a possible alteration in the reward system. Reductions in the rewarding value of abused drugs are associated with increased self-administration, which implies that early nicotine exposure might increase the risk for subsequent substance abuse problems.

Studies in Chapter 7 will examine the effects of nicotine on cocaine-induced reward and sensitivity. Several groups have begun to explore the theory that adolescent nicotine exposure may alter cocaine-induced effects later in development, but results have been inconsistent to date. Kelley and Rowan (2004) found that C57BL/6J mice demonstrated a decrease in cocaine-induced reward as measured by CPP after 25 days of adolescent nicotine exposure. However, they also noted that this exposure led to an increase in cocaine's motor activating effects. In rats, a study by McQuown et al. (2006) showed that a low dose of nicotine treatment for four days in adolescence enhanced the reinforcing effects of cocaine in an i.v. self-administration model using a FR1 schedule. Similarly, rats given nicotine from PND 35 to 44 demonstrated an enhancement of cocaine-induced reward using a CPP paradigm (McMillen et al. 2005). Based on the literature, it appears that nicotine exposure can lead to alterations in cocaine sensitivity. However, studies have limited implications since measures have not been evaluated under the same conditions and specific characterization of dose effects, duration of exposure, and route of administration have not yet been

investigated. The reasons for this "cross-sensitivity" are yet to be fully understood, but mechanisms such as dopamine neurotransmission could be involved.

G. Dissertation Objectives

The work in this dissertation addresses a three part hypothesis which will contribute to the understanding of age-dependent differences in nicotine dependence. To date, few studies have examined adolescence as a heightened period for vulnerability to drug addiction. However, adolescent drug abuse is becoming a large problem in the United States as teenagers are developing and maturing at a faster pace than they did 10 to 20 years ago. More young teenagers and even pre-teens are experimenting with drugs and this experimentation often begins with cigarette smoking. These studies demonstrate a full characterization of nicotine dependence in various age groups and both sexes, examine behavioral and molecular mechanisms which may underlie these differences, and investigate the effects of adolescent smoking on future, and perhaps long-term, drug abuse.

Overall, we hypothesized that vulnerability to nicotine in adolescence is due to a shift in the balance between two key components of nicotine dependence, namely reward and withdrawal, and that this shift is due to nicotine-induced, region-specific changes in mesolimbic reward pathways. Furthermore, we predicted that nicotine exposure in adolescence would lead to long lasting changes in nicotine behavior and dependence as well as the rewarding mechanisms of other drugs of abuse.

Our first specific aim was to characterize the behavioral effects of nicotine using two key measures of nicotine dependence: reward and withdrawal. These studies were

conducted in both the male and female sex. Based on preliminary data and previous literature, we hypothesized that adolescents would demonstrate increased vulnerability to nicotine dependence as compared to adults and that this vulnerability would be sex-dependent.

The second specific aim was to examine the behavioral and molecular mechanisms that may be involved in nicotine dependence pathways using both *in vivo* and *in vitro* techniques. Our *in vivo* studies focused on characterizing levels of acute sensitivity and tolerance to nicotine between adult and adolescent mice. After that, we aimed our *in vitro* studies at the initial molecular target of nicotine: neuronal nicotinic acetylcholine receptors. To this aim, we investigated changes in the function and quantity of receptors using rubidium efflux assays and nAChR binding studies, respectively. We also examined differences in levels of nicotine-induced dopamine release from both ages; an important measurement of nicotine reward and reinforcement. Our hypothesis was that adolescents would exhibit an increase in either receptor number or function, if not both, which would contribute to our understanding of increased nicotine addiction vulnerability.

The third and final specific aim was to examine whether adolescent nicotine exposure resulted in long-lasting changes in levels of nicotine reward and withdrawal. Both dose and duration of nicotine exposure were investigated since patterns of exposure to nicotine have been shown to be an important factor in becoming dependent (McNeill 1991). In addition to investigating this type of exposure on nicotine dependence, we also examined the effects of adolescent nicotine exposure on cocaine

sensitivity. Rewarding effects, changes in locomotor activity, and locomotor sensitization to cocaine were evaluated. For this aim, we hypothesized that exposure to nicotine during early adolescence would cause persistent changes in both nicotine- and cocaine-related behavior in adulthood. Specifically, perception of nicotine- and cocaine-induced reward will be enhanced due to long-lasting effects on the mesocorticolimbic reward pathway.

In summary, data from this study are the first to fully evaluate a battery of nicotine dependence models in the same setting and conditions in different sexes and ages. Furthermore, it investigated possible *in vivo* and *in vitro* mechanisms that could be used in developing more effective smoking cessation strategies aimed at specific audiences. Lastly, these studies provided new insight into the risks of adolescent smoking. We have shown that experimentation with cigarette smoking may have long-lasting effects on future drug dependence; thus demonstrating the importance of effective prevention messages and smoking deterrents that should be made available to youth.

BEHAVIORAL ASSESSMENT OF ADOLESCENT AND ADULT MICE IN NICOTINE DEPENDENCE MODELS OF REWARD AND WITHDRAWAL

A. Introduction

While a few studies have investigated various aspects of nicotine dependence in both adult and adolescent rodents, there is no comprehensive study examining multiple features of nicotine dependence under the same experimental conditions. Since behavioral effects of nicotine may vary with the paradigm employed, it is important to conduct studies where various behavioral responses are evaluated in the same setting and in parallel. The goal of this study was to fully characterize both age- and sex-related differences in nicotine dependence models of reward and withdrawal in mice.

Nicotine elicits consistent rewarding effects and withdrawal signs which are characteristic of dependence. Conditioned place preference (CPP) procedures have been widely used as a measure of the potential rewarding effects of many different psychoactive drugs (Bardo et al. 1995; Tzschentke 1998). Several groups have established a nicotine-induced CPP in rodents (Biala et al. 2003; Walters et al. 2005). Unlike other models, such as self-administration, this model does not directly measure drug reinforcement; rather it is a measure of a preference for a context which is associated with the drug stimulus. However, as demonstrated throughout the literature, there is a reasonable concordance between drugs that produce a CPP and drugs that are self-administered (Bardo and Bevins 2000) and data from this method serve to complement self-administration data. CPP is advantageous because the animals are

tested in a drug-free state and it does not require extensive surgical procedures which are stressful to the animal.

In adult rodents, nicotine withdrawal has been well-characterized. Malin et al. (1992, 1994) demonstrated increased numbers of abstinence signs following nicotine cessation and following administration of nicotinic antagonists in rats. Similar effects have been noted in mice. An increase in withdrawal signs was reported in a number of studies (Isola et al. 1999; Damaj et al. 2003) as well as hyperalgesia (Damaj et al. 2003) upon both spontaneous withdrawal (no antagonist) and precipitated withdrawal (antagonist) testing in mice of different genetic backgrounds.

Only a limited number of studies have investigated these two aspects of nicotine dependence in the adolescent rodent. Vastola (2002) reported that in the CPP paradigm only rats conditioned during adolescence showed preference to nicotine; however, to date, there have been no studies which have investigated this model of reward in the mouse. Adolescent nicotine withdrawal has been examined in a rat model as well. O'Dell et al. (2006) reported decreased somatic signs in the adolescent rat as compared to the adult rat. In addition, Shram et al. (2006) demonstrated that early adolescent rats do not develop avoidance to saccharin solutions which have been paired with nicotine while adults do develop this avoidance. The present study will examine both reward and withdrawal paradigms in the mouse model in order to extend the current findings.

B. Methods

General methodology for all studies

Choice of Age, Sex, and Strain

As previously mentioned in Chapter 1, adolescence in the rodent is defined using various factors informative of developmental transitions in human adolescents. Our studies will use the standard divisions that have been defined by previous studies in rodents: early- (PND 21-34), mid- (PND 35-46) and late- (PND 47-59) adolescence (Spear 2000 and Laviola 2003).

ICR mice were used in all studies. This strain is an outbred stock with a fast growth rate which is conducive to our experimental procedures. It has been used extensively in toxicology and pharmacology studies. All animals were ordered from Harlan Laboratories (Indianapolis, IN). We are aware that litter effects, particularly in the adolescent age, may present confounds in our data analysis. To ensure that this was not the case, we requested that our animals were obtained from different litters. We also ordered animals at different times to minimize this issue. Animals were maintained in an American Association for Accreditation of Laboratory Animal Care approved facility and the study was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

This chapter focuses on the behavioral characterization of nicotine dependence in both male and female mice. Even though sex differences were apparent, further studies focused solely on the male sex. We felt that it was beyond the scope of this

project to properly examine the changes in hormonal levels during adolescence and decided to center our research on male mice so that we could fully characterize this sex.

Drugs

(-)-Nicotine bitartrate and mecamylamine hydrochloride were purchased from Sigma Chemical Company (Milwaukee, WI). All doses are expressed as free base.

Nicotine-induced conditioned place preference

Place conditioning boxes consisted of two distinct sides (20 cm X 20cm X 20 cm). A partition separated the two sides with an opening that allowed access to either side of the chamber, and this partition could be closed off for pairing days.

Handling habituation: On Wednesday – Friday of the week prior to the start of the place conditioning procedure, mice in the CPP studies were handled once per day for approximately two min each. Handling experience plays an important role in the ability of nicotine to produce a conditioned place preference (Grabus et al. 2006).

Preconditioning Phase: On day 1, animals were placed in the boxes and allowed to roam freely from side to side for 15 min, and time spent in each side was recorded. These data were used to separate the animals into groups of approximately equal bias.

Conditioning Phase: Animals were paired for 20 min with the saline group receiving saline in both sides of the boxes and drug groups receiving nicotine (0.05, 0.1, 0.5, 0.7 or 1 mg/kg) on one of the sides and saline on the opposite side. Drug-paired sides were randomized among all groups. Conditioning lasted for 3 days, with animals in the drug group, receiving drug each day.

Test Phase: On the test day, no injections were given. Time spent on each side was recorded, and data were expressed as time spent on drug-paired side minus time spent on saline-paired side. A positive number indicated a preference for the drug-paired side, while a negative number indicated an aversion to the drug-paired side. A number at or near zero indicated no preference for either side.

Mecamylamine-Precipitated Withdrawal Studies

Naïve mice were implanted with osmotic mini-pumps filled with either saline or nicotine (48 mg/kg/day) on day 1. The mini-pumps were surgically implanted s.c. under sterile conditions with pentobarbital anesthesia (35 mg/kg, i.p.). An incision was made in the back of the animals, and a pump was inserted. Animals were sutured and allowed to recover before being returned to their home cages. On the morning of day 8, mice were injected s.c. with 2.0 mg/kg of mecamylamine, a nicotinic antagonist. Ten minutes following injection with the antagonist, mice were tested for withdrawal signs in the following manner: 5 min for anxiety-like behavior (on the elevated plus maze described below), 20 min observation of somatic signs (paw tremors, head shakes, backing, body tremors, ptosis), hyperalgesia (hot-plate test), and 30 min in locomotor activity chambers.

Elevated plus maze. The EPM is an apparatus consisting of two closed arms and two open arms. The mouse is placed in the center of the maze and allowed to roam freely between the open and the closed arms for 5 min. The number of seconds the mouse spends in the open arms is counted by a counting device attached to beams located on

both arms and in the middle of the plus maze. Scores are based on time spent in the open arms as an indication of anxiety-like behavior.

Hot-Plate Test. Supraspinal antinociception was assessed by the hot-plate method. Briefly, each mouse was injected s.c. with nicotine and tested 5 min after injection. Mice were placed on a hot plate (Thermostat Apparatus, Columbus, OH) maintained at 55˚C. Latency to reaction time (jumping or paw licking) was recorded. A control response (8-12 sec) was determined for each mouse before treatment and test latency was determined after drug administration. A maximum latency of 40 sec was imposed. Antinociceptive response was calculated as %MPE, where %MPE = [(test - control)/(40 - control) x 100].

Locomotor Activity. Mice were placed into individual Omnitech photocell activity cages (Columbus, OH; 28 x 16.5 cm) 10 min after s.c. administration of nicotine. Interruptions of the photocell beams (two banks of eight cells each) were then recorded for the next 30 min. Data are expressed as number of photocell interruptions.

Spontaneous Withdrawal Studies

Naïve mice were implanted with osmotic mini-pumps filled with either saline or nicotine (48 mg/kg/day) for 7 days (as described previously). On day 8, mice were lightly anesthetized using ether and mini-pumps were removed. A small incision was made on the back of the neck in order to remove the mini-pump and the wound was closed with a suture. Twelve hours following removal of the mini-pump, mice were evaluated with the same withdrawal measures described above.

Repeated Injection Withdrawal Model

Naïve mice were injected s.c. with 2.0 mg/kg of nicotine three times a day for four days (injections were given at 8:00am, 12:00pm, and 4:00pm). On the morning of day 5, mice received 2.0 mg/kg of mecamylamine s.c. and were evaluated 10 min later in the same withdrawal measures previously described.

Statistical analysis

Statistical analysis of all behavioral studies was performed with mixed-factor ANOVA with post-hoc Tukey's test when appropriate. P-values of <0.05 were considered to be statistically significant.

C. Results

Nicotine-induced Conditioned Place Preference

The ability of nicotine to produce an effect in conditioned place preference in both ages and sexes is presented in Fig. 2. As expected, adult and early adolescent animals injected with saline in both chambers, showed no preference for either chamber. Compared to saline controls, male adult mice conditioned with the intermediate dose of 0.5 mg/kg nicotine showed a significant place preference. There were no significant preferences seen with lower doses of 0.05 or 0.1 mg/kg, and the effect disappeared at the higher doses of 0.7 and 1.0 mg/kg nicotine. In contrast, nicotine induced a significant place preference in adolescent mice at low doses of nicotine (0.05 and 0.1 mg/kg) as well as at the 0.5 mg/kg dose. Similar to the adult mice, the effect disappeared at the higher doses of 0.7 and 1.0 mg/kg nicotine.

In female mice, a 2 x 2 ANOVA (age x dose) revealed a significant interaction. Upon further post-hoc analysis, it was found that compared to saline controls, adult

mice conditioned with the doses of 0.7 and 1.0 mg/kg nicotine showed a significant place preference; whereas no significant preferences developed with lower doses of 0.1 or 0.5 mg/kg. In contrast, nicotine produced a significant CPP in adolescent mice at the intermediate dose of 0.5 mg/kg; an inactive dose in adults. Moreover, adolescents experienced CPP at a narrow dose range and the dose-response curve is shifted to the left. Locomotor activity was also recorded and did not differ between the age groups. (Adults: 988 ± 32, Adolescents: 1012 ± 44).

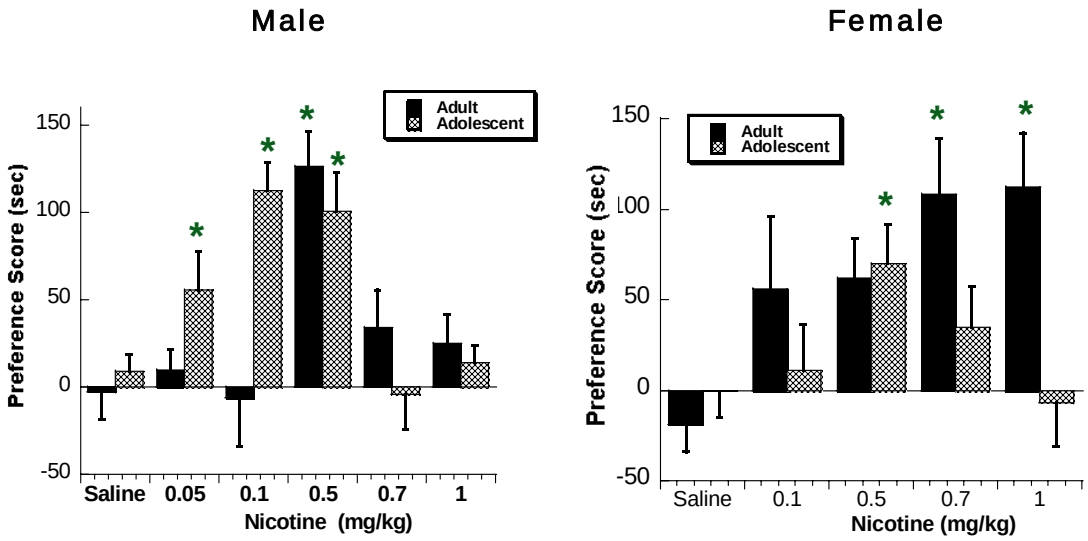

Figure 2. Nicotine-induced conditioned place preference in male and female mice. Adult (PND 70) and early adolescent (PND 21) were injected s.c. with various doses of either saline or nicotine. Positive scores indicate a preference for nicotine while negative scores are indicative of aversion to the drug. Scores at or near zero indicate neither preference nor aversion. Each bar represents the mean ± SEM of 8-9 mice. *p<0.05 from saline group.

Age-Dependent Acquisition of Conditioned Place Preference

One possible explanation for the enhanced preference seen in adolescent mice is the rate of CPP acquisition. It has been suggested that adolescents have an increased rate of learning as compared to adults. To examine this possibility, we conducted a CPP study in which subsets of mice were conditioned with nicotine for either 1, 2, or 3 sessions and were subsequently tested for CPP the following day using a dose of 0.5 mg/kg nicotine since this dose was active in both ages in previous experiments. This experiment was only performed in male mice as this sex was determined to be the focus of our studies. Figure 3 shows the results of this study which demonstrate that both adult and adolescent mice acquired a significant preference for nicotine after three conditioning sessions. Neither one nor two sessions elicited a preference for either compartment.

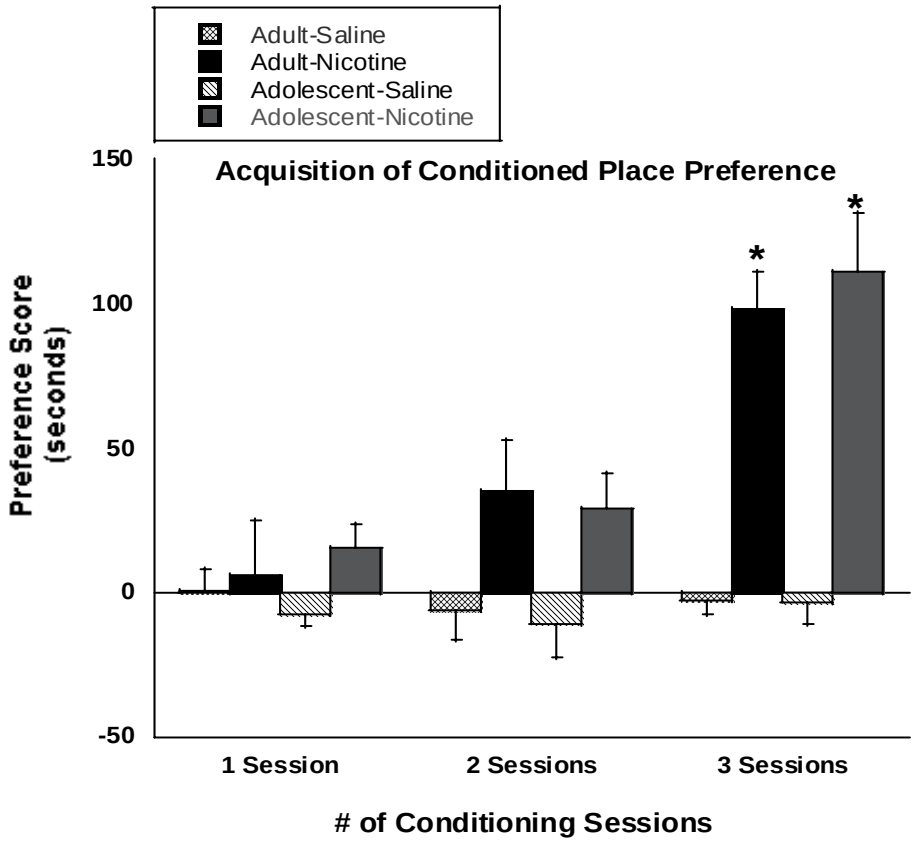

Figure 3. The rate of CPP acquisition in adult and adolescent mice. Male ICR were conditioned for 1, 2, or 3 sessions with 0.5 mg/kg nicotine and were subsequently evaluated for nicotine-induced preference. Bars represent the mean ± SEM of 6-8 mice. * $p<.05$ as compared to respective saline control.

Precipitated Withdrawal Model

Males

Mecamylamine challenge to animals implanted with nicotine-filled mini-pumps represents a highly reproducible precipitated withdrawal syndrome. Measures of precipitated withdrawal in adult and adolescent male mice are shown in Fig. 4. Two types of withdrawal signs were evaluated: physical (somatic signs, hyperactivity, and hyperalgesia) and affective (anxiety-like behavior in the plus maze). In males, adolescent mice displayed fewer withdrawal signs in all four measures as compared to adults. Adolescents displayed a lower number of somatic signs (Fig. 4a) as compared to adults. In the elevated plus maze (Fig. 4b), only adults displayed withdrawal as indicated by a significant decrease in time spent in the open arms of the maze, an indication of anxiety-like behavior. Similarly, in the hot-plate test (Fig. 4c) and locomotor activity test (Fig. 4d), only adult mice displayed hyperalgesia and hyperactivity respectively.

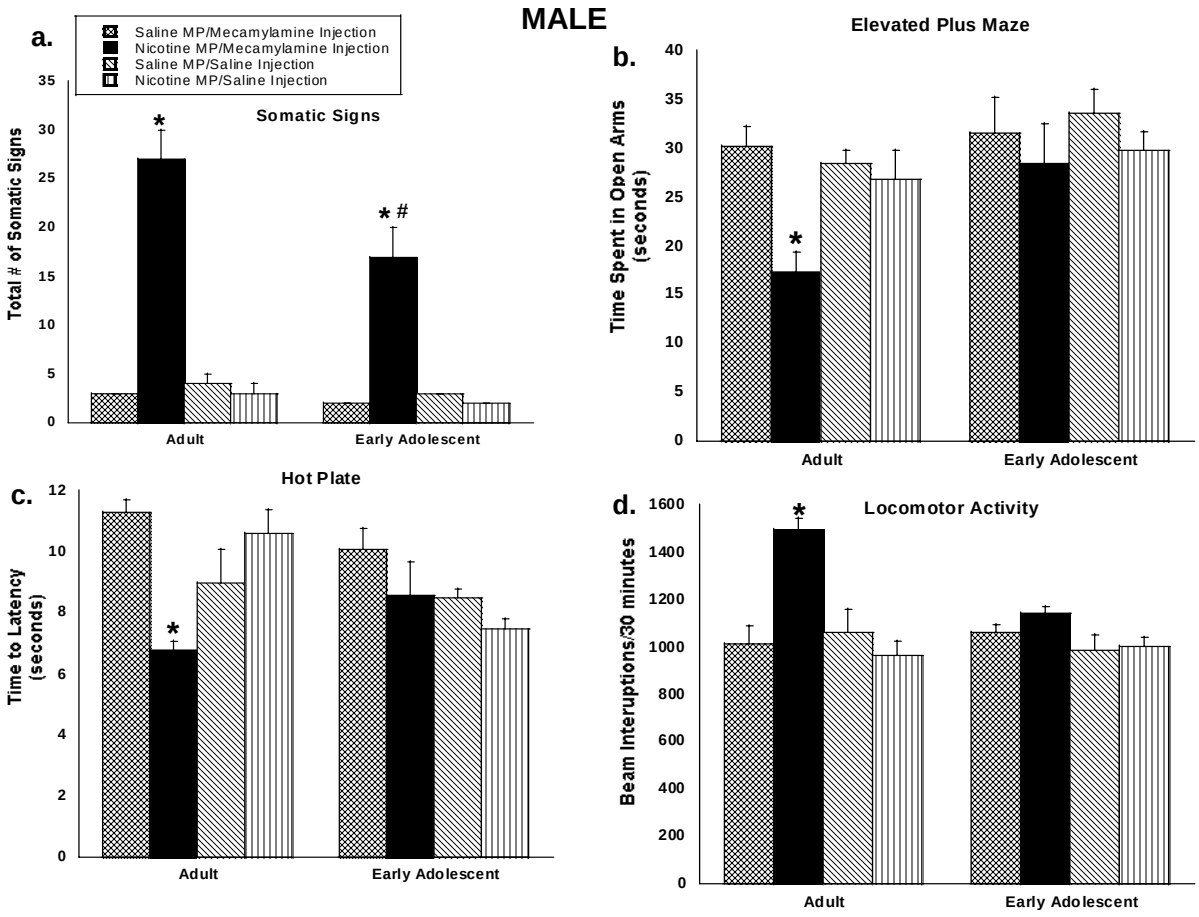

Figure 4. Mecamylamine-precipitated withdrawal in adult (PND 70) and adolescent (PND 21) male mice. Mice were chronically infused with nicotine at 48 mg/kg/day or saline for 7 days. On day 8, mice were injected with 2.0 mg/kg of mecamylamine or saline s.c. to precipitate withdrawal and evaluated in four tests: (a) somatic signs, (b) elevated plus maze, (c) hot plate analgesia test, and (d) locomotor activity. * p<0.05 from saline group and #p<0.05 from adult nicotine treatment. Each point represents the mean ± S.E. of 12 mice. MP=mini-pump.

Females

Female mice displayed an opposite trend in withdrawal. In somatic signs (Fig 5a), results showed that there was a significant 2 way interaction (age x treatment), indicating that both ages demonstrated significant withdrawal signs, but the intensity of withdrawal was age-dependent. Indeed, adolescent females displayed significantly higher somatic signs as compared to adults as determined through post-hoc analysis. In the hot plate analgesia test (Fig. 5c), there was no significant interaction. However, adolescents with nicotine mini-pumps displayed a significant attenuation in analgesia upon treatment with mecamylamine, indicative of withdrawal, while adults failed to do so ($p=.08$). Adolescents also showed significant withdrawal in hyperactivity (Fig. 5d), while adult mice did not, but no significant interaction was found.

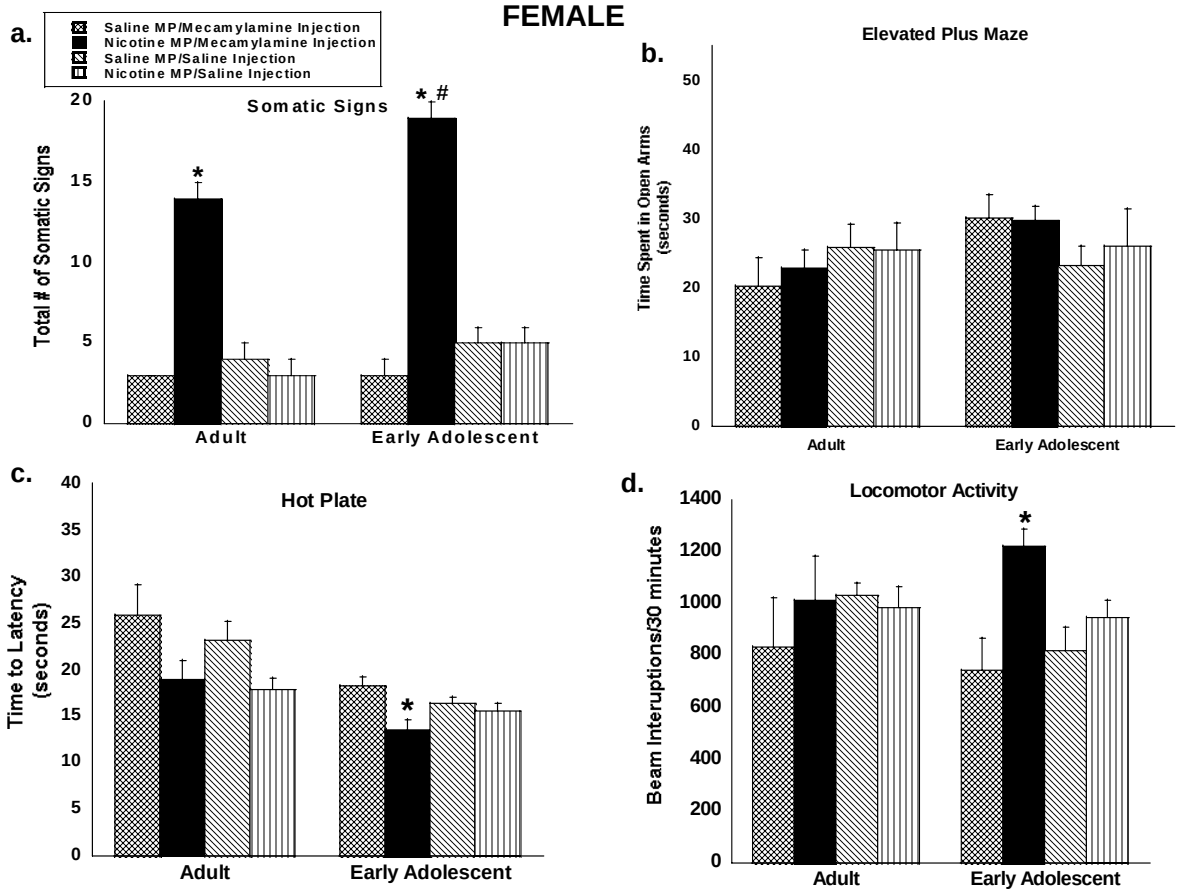

Figure 5. Mecamylamine-precipitated withdrawal in adult (PND 70) and adolescent (PND 21) female mice. Mice were chronically infused with nicotine at 48 mg/kg/day or saline for 7 days. On day 8, mice were injected with 2.0 mg/kg of mecamylamine or saline s.c. to precipitate withdrawal and evaluated in four tests: (a) somatic signs, (b) elevated plus maze, (c) hot plate analgesia test, and (d) locomotor activity. * p<0.05 from saline group and #p<0.05 from adult nicotine treatment. Each point represents the mean ± S.E. of 12 mice. MP=mini-pump.

Spontaneous Withdrawal Model

Males

While the mecamylamine-precipitated withdrawal model revealed clear differences between adolescents and adults, the question arises as to whether adolescents and adults have identical sensitivities to mecamylamine. In order to investigate this possibility, spontaneous withdrawal studies were carried out. In the spontaneous withdrawal model, mini-pumps were removed on day 8 and no drugs were used to elicit withdrawal behaviors. As shown in Fig. 6, both adult and adolescent male mice displayed a significant increase in the number of somatic signs after chronic treatment with nicotine; however, adolescent mice had significantly fewer of these signs as compared to adults (Fig. 6a). Results were similar for the hot-plate (Fig. 6c) and locomotor activity (Fig. 6d) tests which confirmed the data from the precipitated withdrawal model. Only adult mice displayed affective withdrawal signs in the spontaneous model as shown by the decrease in time spent in the open arms of the elevated plus maze (Fig. 6b).

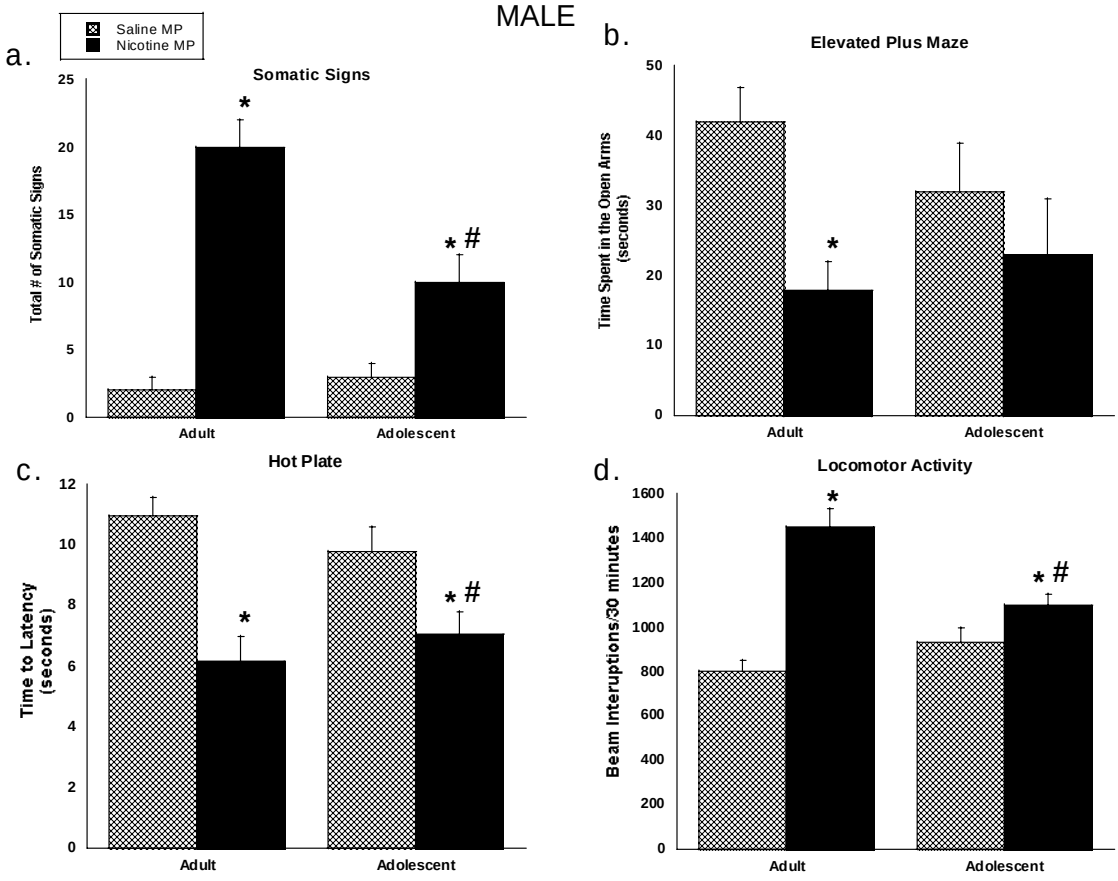

Figure 6. Spontaneous withdrawal in adult (PND 70) and adolescent (PND 21) mice. Male mice were chronically infused with nicotine at 48 mg/kg/day (dark bars) or saline (cross-hatched bars) for 7 days. Twelve hours after mini-pumps were removed, mice were tested for: (a) somatic signs and (b) elevated plus maze. Each point represents the mean ± S.E. of 12 mice. * $p<0.05$ from saline group and # $p<0.05$ from adult nicotine treatment. MP = mini-pump

Females

As shown in Fig. 7a, females also exhibited significant somatic signs of withdrawal, but adolescents had a significantly greater number of signs than did adults. Furthermore, only adolescents displayed significant hyperalgesia and hyperactivity during the withdrawal testing (Fig. 7c and 7d) which is consistent with data from precipitated withdrawal studies.

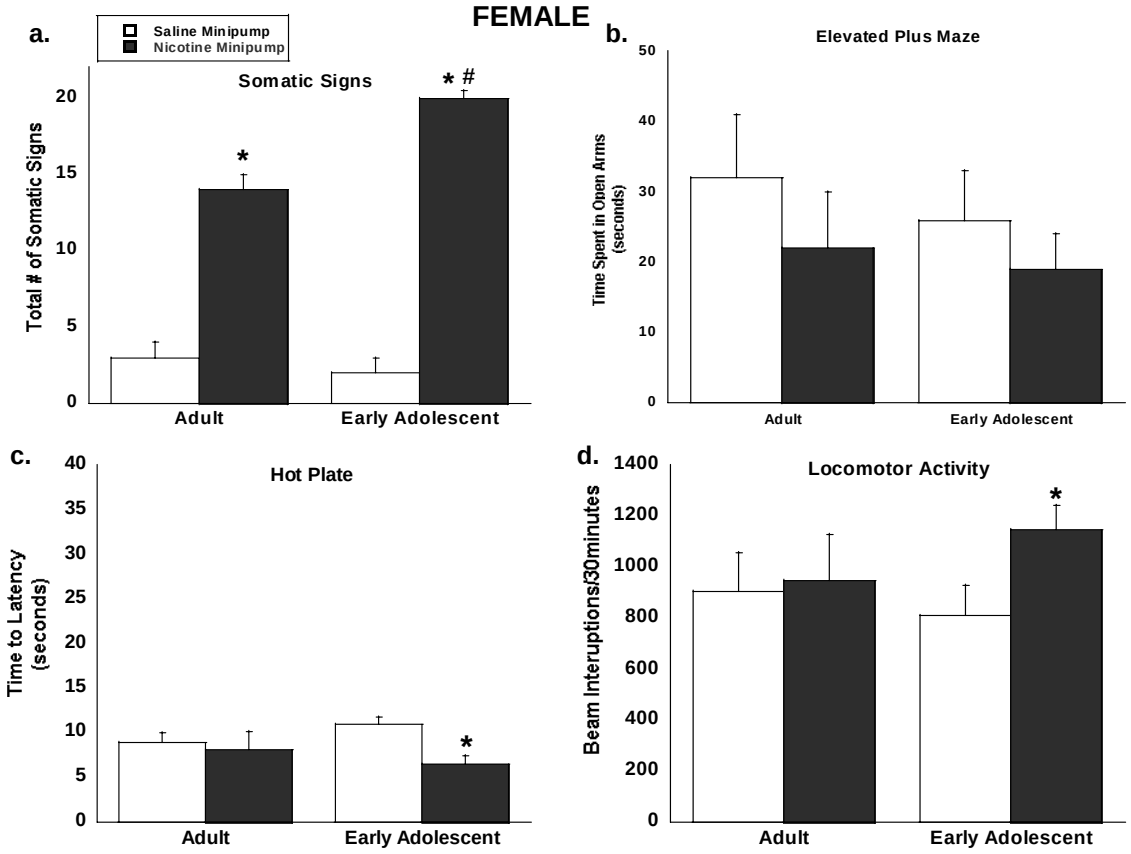

Figure 7. Spontaneous withdrawal in adult (PND 70) and adolescent (PND 21) female ICR mice. Mice were chronically infused with nicotine at 48 mg/kg/day (grey bars) or saline (open bars) for 7 days. Twelve hours after mini-pumps were removed, mice were tested for: (a) somatic signs and (b) elevated plus maze (c) hot plate analgesic test, and (d) locomotor activity. Each bar represents the mean ± S.E. of 12 mice. * $p<0.05$ from saline group and # $p<0.05$ from adult nicotine treatment. MP = mini-pump

Repeated Injection Withdrawal Model

Males

One of the limitations of mini-pumps is the inability to alter drug delivery during the exposure period, particularly when adolescents and adults are gaining weight at different rates. Therefore, we replicated the above studies with repeated injections based on the daily weights of the animals. The repeated injection model confirmed our previous withdrawal results with mini-pumps as shown in Fig. 8. Hyperactivity is demonstrated only by adult mice in the locomotor activity test (Fig. 8d). Furthermore, in the hot-plate test (Fig. 8c), adult mice displayed hyperalgesia while adolescent mice failed to exhibit this effect. In the affective sign of withdrawal, only adults exhibited anxiety-like behavior in the elevated plus maze (Fig. 8b).

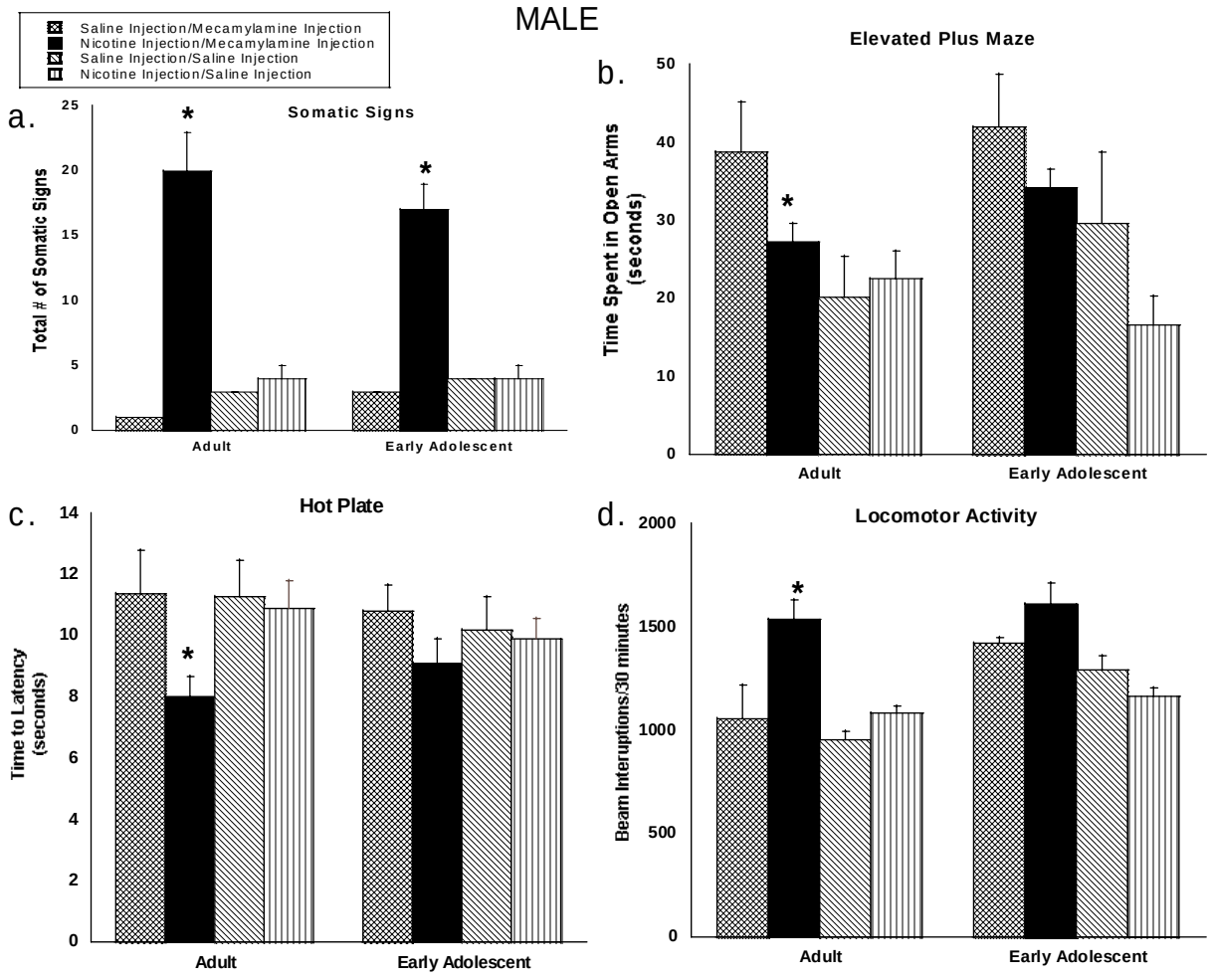

Figure 8. Withdrawal following repeated injections in adult (PND 70) and adolescent male (PND 21) mice. Mice were injected with saline or 2.0 mg/kg of nicotine s.c. three times a day for four days. Sixteen hours following the last injection, mice were injected with 2.0 mg/kg of mecamaylamine or saline s.c. and evaluated for: (a) somatic signs (b) elevated plus maze (c) hot plate analgesia and (d) locomotor activity. Each point represents the mean ± S.E. of 10 mice. * $p<0.05$ from saline group.

Females

The repeated injection model was also conducted in female mice as shown in Fig. 9. As with the precipitated model, adolescent mice displayed a significant increase in somatic signs as compared to adults (Fig. 9a). Unexpectedly, in the elevated plus maze (Fig. 9b), adolescents showed a decrease in anxiety-like behavior while adults had no change from control animals. In the hot-plate test, adolescent mice displayed significant hyperalgesia (Fig. 9c) while adults showed no indication of withdrawal. Surprisingly, no hyperactivity was seen in the locomotor activity test (Fig. 9d). The overall intensity of this test was lower than normal which may be attributable to stress induced by the multiple injections used in this model.

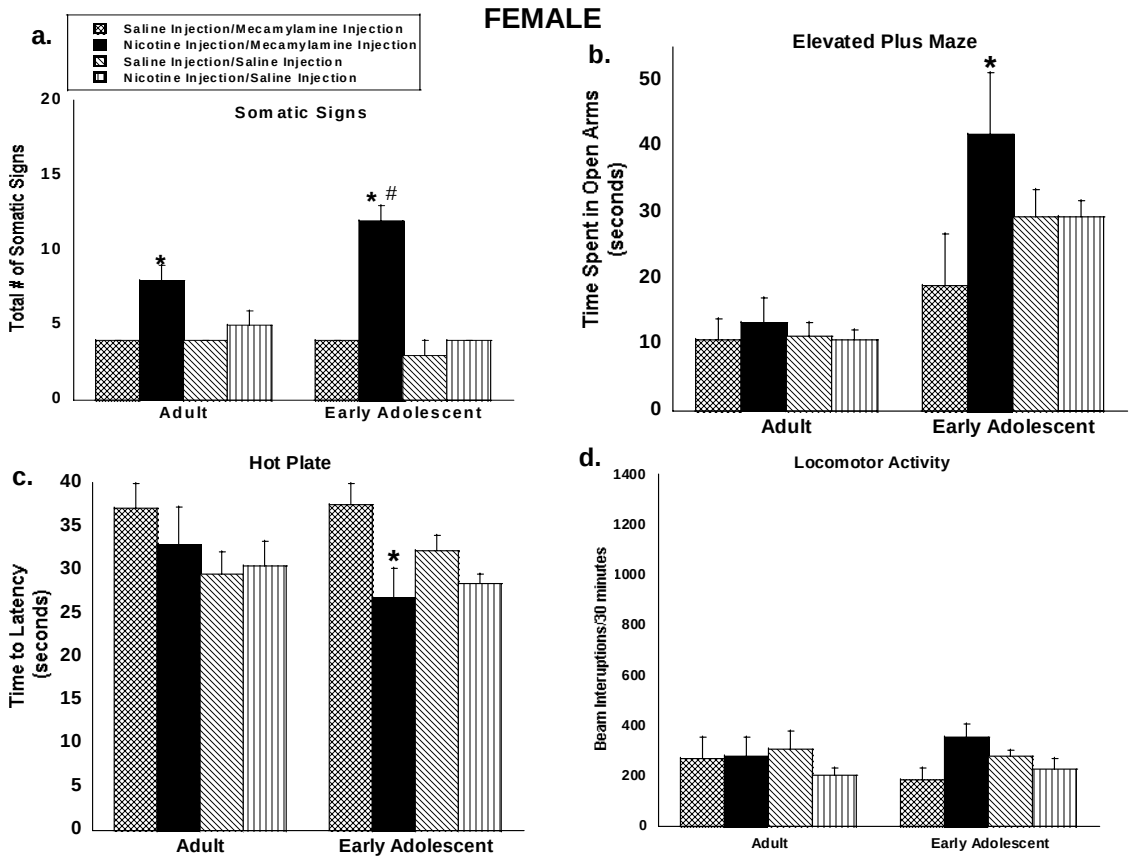

Figure 9. Withdrawal following repeated injections in adult (PND 70) and adolescent (PND 21) female ICR mice. Mice were injected with saline or 2.0 mg/kg of nicotine s.c. three times a day for four days. Sixteen hours following the last injection, mice were injected with 2.0 mg/kg of mecamaylamine or saline s.c. and evaluated for: (a) somatic signs and (b) elevated plus maze (c) hot plate analgesic test, and (d) locomotor activity. Each bar represents the mean ± S.E. of 10 mice. * p<0.05 from saline group and # p<0.05 from adult nicotine treatment.

D. Discussion

There is little question that social and environmental factors play significant roles in initiation of tobacco use. While these factors contribute to progression from use to addiction, there is undoubtedly a biological basis as well. Given that nicotine use often begins in adolescent years, it is reasonable to speculate that developmental changes are important contributors. These changes may impact important aspects of nicotine dependence such as reward and the magnitude of withdrawal. The goal our first study was to investigate both age- and sex-related differences in nicotine dependence using behavioral rodent models. From our results, it appears that reward and withdrawal are two key components of adolescent nicotine dependence which may have implications in adolescent vulnerability to nicotine addiction. For simplicity, the following discussion will discuss age differences in each sex individually before addressing the issue of sex differences.

Our data show that adolescent male mice are more sensitive to nicotine's rewarding effects than adult male mice in the CPP model. Several factors could explain the differences in nicotine potency; that is to say that different concentrations of nicotine may result in certain effects at one age while not at another age. One such factor is differences in motor function between adult and adolescent mice. However, locomotor activity data suggests no differences in motor function (Figure 10). Alternatively, the conditioning session and duration might have been optimal for adolescents but not for adults at a low dose of nicotine. Varying the duration of the sessions may alter the development of place preference at lower doses in adults and

adolescents. However, previous studies have tested a variety of conditioning sessions and durations and have not identified this as a confounding factor (Belluzzi et al. 2004). An additional factor when using CPP involves contextual learning and memory. It is possible that adolescents display increased learning as compared to adults. Our acquisition study (Figure 3) rules out this possibility in that we showed no difference in the rate of CPP acquisition between adults and adolescents. Finally, it is possible that pharmacokinetic differences between adult and adolescent rodents could explain the different potencies. If this is the case, adolescents are reported to have increased metabolism (Trauth et al. 2000); this however would translate into a decrease, not an increase, in the rewarding effect of nicotine. Given that metabolism is not a largely contributing factor, our data suggest that pharmacological factors are responsible for the enhanced rewarding effect from nicotine conditioning in adolescent rodents.

Data from our withdrawal study showed that male adolescent mice displayed decreased withdrawal signs in three different somatic measures and one affective measure. However, results should be interpreted with caution because of several possible confounding factors. Although the use of mini-pumps allows for a minimal amount of stress to be placed on the animal, it will not take into account the fact that adolescent animals display a growth rate much faster than that of adults. Since the dose of nicotine is based on body weight at the beginning of the experiment, it could be argued that the younger mice are not receiving a dose of nicotine that is consistent with that of the adult. For this reason, we also evaluated withdrawal using a repeated

injection model that was based on the animal's daily weight. In both studies it was found that withdrawal signs were attenuated in adolescent animals.

Another factor that could be playing a role is that adults and adolescents could have differing sensitivities to mecamylamine. In order to investigate this possibility, we conducted a spontaneous withdrawal experiment in which mecamylamine was not used to precipitate withdrawal. Again, similar and consistent results were found in this study with adolescent animals displaying a decrease in the intensity of withdrawal signs. Collectively, these studies suggest a minor role for the two above factors in the withdrawal differences.

Overall, our data have significant implications. First, we have shown that adolescents, when given the same level of nicotine as adults, express withdrawal signs which are an important observation from a clinical perspective. Although the intensity of nicotine withdrawal is less than that of the adult, this finding confirms work in clinical studies showing that adolescent smokers exhibit signs of nicotine dependence. It is also interesting to note that adolescent smoking behavior is not consistent with that of adults and it is likely that their actual nicotine intake is lower. In this regard it is difficult to make a valid assessment of how much withdrawal symptoms contribute to dependence for adolescents. In addition to withdrawal signs, we have shown that during adolescence positive rewarding effects of nicotine are enhanced in the male sex. It could be argued that these positive effects contribute more to the enhanced vulnerability to nicotine addiction. During the adolescent period there is a high desire

for immediate positive reinforcement without proper assessment of risk which suggests that adolescence may be a critical period for the development of nicotine addiction.

Female studies in nicotine dependence showed characteristically different results than those from male studies. It is well known that the incidence of smoking among women has changed dramatically over the past few decades. Moreover, it has become clear in recent times that females experience considerable difficulty in quitting smoking (Perkins et al. 1999; Field and Duka 2004; Leventhal et al. 2007). The goal of this study was to identify the properties of nicotine that might explain differences in adolescent and adult female behavioral responses to this drug.

The results from our CPP study show age-dependent differences in nicotine-induced reward sensitivity in females. Significant preference was observed at a dose of 0.5 mg/kg in adolescent mice while adult mice displayed rewarding effects at 0.7 and 1.0 mg/kg demonstrating differences in the range of preference. It appears that adolescents display an enhanced sensitivity to nicotine's rewarding effects at lower doses than adults. However, this may only play a minor role in overall dependence due to the narrow dose-response curve. Once again, nicotine-induced differences in locomotor activity between adult and adolescent mice may cause the observed differences. However, as stated previously, data for locomotor activity were collected and no significant differences were found (Figure 11). It is also possible that pharmacokinetic differences between adult and adolescent rodents could explain the different potencies. It is well-established in adult rodents that sex and age can affect nicotine metabolism. Although some reports suggest that adolescents have an

increased metabolism (Adriani et al. 2002; Klein et al. 2004), our data suggest that this change in metabolism does not play a major role in the differences seen in our studies since the acute effects are not consistent across all measures (this is will be further discussed in Chapter 3).

Nicotine withdrawal studies in the female sex indicate that adolescents have increased physical withdrawal symptoms (somatic signs, hyperalgesia, and hyperactivity) as compared to adult females. However, no differences in affective withdrawal signs (anxiety-like behavior) were observed. This enhanced withdrawal intensity is likely to adversely affect adolescents who are attempting to quit smoking. In agreement with our rodent studies, clinical findings report that women are less likely to quit smoking successfully due to high withdrawal effects (Leventhal et al. 2007). For these reasons, treatment strategies that focus on alleviating these negative withdrawal symptoms are likely to be the most effective in female smokers, particularly in adolescents. A possible cause for this increase in withdrawal intensity is the higher rate of metabolism in adolescents (Trauth et al. 2000), however, as previously stated, metabolism is not likely to be the most important contributor to this difference. In addition, data has shown that sex hormones are able to modulate the effects of nicotine which may contribute to differences seen between male and female rodents (Damaj 2001). However, this possibility is also unlikely given that female adolescents are not sexually mature at this stage of development.

The current study, as well as previous research (Trauth et al. 1999; Adriani et al. 2004; O'Dell et al. 2006; Shram et al. 2006) suggests that adolescence is a critical

period for vulnerability to nicotine dependence in both male and female adolescents. Nicotine dependence is based on the balance between the positive and negative effects of nicotine. Certain theories of nicotine dependence have suggested that some people smoke in order to cope with negative withdrawal symptoms, while others smoke for reward and pleasure. Our data suggest that negative withdrawal signs are more strongly associated with nicotine dependence than rewarding effects in female adolescents. However, in males it appears that reward may play a larger role. Development of treatment strategies that are tailored to youth are needed in order to combat the growing problem of adolescent smoking. It is important to note that data from this study show that an opposite association was seen in males and females suggesting that smoking cessation therapies will not necessarily be equally efficacious for each sex at an early age. Furthermore, a comparison of adult withdrawal reveals that males exhibit a greater intensity of nicotine-induced withdrawal signs as compared to females, but also experience enhanced positive and rewarding effects of nicotine.

Taken together, these studies have important implications for the mechanisms of nicotine dependence in adolescence. Several behavioral and molecular mechanisms may explain these differences. Differences in activation, function, and regulation of various targets of nicotine, such as nAChRs, are likely to be involved. We decided to investigate these possibilities by using both *in vivo* and *in vitro* approaches.

It is possible that there are innate differences in the acute sensitivity to nicotine between adults and adolescents. Furthermore, differences in tolerance could help to explain our behavioral observations. Therefore we examined these two aspects of

dependence using *in vivo* models. Additionally, it is possible that functional properties, distribution, and number of nAChRs differ between adult and adolescent rodents. It is also possible that nicotine exposure during early adolescence is altering the neuronal pathways which are still developing in young animals. For example, the dopaminergic system is still developing during the adolescent period (Spear 2000). Many studies have shown that upon activation of nAChRs, dopamine is released and can act to modulate rewarding effects (Wonnacutt 1997). It is possible that levels of dopamine release differ in the adult and adolescent, a factor that may contribute to the differences seen in levels of reward using the CPP model. In order to answer these questions, we took an *in vitro* approach and used a variety of molecular assays to investigate these targets.

Indeed, current research implies that adolescence is a critical period for acquiring nicotine dependence. Since adolescent smokers have been shown to have a more difficult time quitting than smokers who begin in adulthood (Colby et al. 2000), it is critical to understand why this timeframe is especially vulnerable to addiction. A more in depth investigation of these behavioral and molecular mechanisms will be further addressed in Chapters 3 and 5.

IN VIVO PHARMACOLOGICAL MECHANISMS INVOLVED IN NICOTINE DEPENDENCE

A. Introduction

Data from Chapter 2 indicated that two key components of nicotine dependence, namely reward and withdrawal, have differing pharmacological profiles in early adolescent and adult mice. Various mechanisms could be involved in these age differences and determining those which play the greatest role will allow a more comprehensive understanding of this behavior. To this aim, we have examined two possible *in vivo* pharmacological mechanisms which are known to be involved in drug dependence: acute sensitivity and tolerance.

Acute sensitivity models are useful in that they provide information on the immediate response to a drug and could reflect differences in nicotine receptor activation and function. In addition, these initial effects can provide insight into the biological systems on which the drug will act as well as any individual differences that may be useful in identifying at-risk individuals before they become addicted to certain drugs.

Tolerance studies usually allow the detection of the capacity of the body to become less responsive to a particular substance; usually after chronic exposure to that substance and could reflect differences in the receptor regulation and consequent signal transduction mechanisms after chronic exposure to a drug. Tolerance is likely to contribute to repeated nicotine use and lead to physical dependence. If adolescent and adult rodents display differences in levels of tolerance or immediate drug sensitivity,

this will provide insight into the underlying behavioral differences in reward and withdrawal models.

Previous studies have shown that nicotine administration elicits several consistent acute effects including hyperalgesia (Marubio et al. 1999), hypoactivity (Clarke 1990; Dwoskin et al. 1999), and hypothermia (Knox et al. 1973). However, tolerance to these initial effects has also been well-documented. Specifically, rodents exposed to chronic nicotine administration show reduced responsiveness to analgesic assays (Damaj and Martin 1993), hypothermia and locomotion (Robinson et al. 1996). It is important to consider these two aspects in explaining variations in levels of nicotine dependence between age groups.

B. Methods

Acute Sensitivity

Mice were given s.c. injections of nicotine and tested in four pharmacological tests: analgesia (two assays, tail-flick and hot-plate), locomotor activity and hypothermia.

Tail-Flick Test. Spinal antinociception was assessed by the tail-flick method of D'Amour and Smith (1941). Briefly, mice were lightly restrained while a radiant heat source was directed onto the upper portion of the tail. A control response (2-4 sec) was determined for each mouse before treatment, and test latency was determined after drug administration. In order to minimize tissue damage, a maximum latency of 10 sec was imposed. Antinociceptive response was calculated as percent maximum possible effect

(% MPE), where %MPE = [(test-control)/(10-control)] x 100. The mice were tested 5 min after injection of nicotine.

Hot-Plate Test. Supraspinal antinociception was assessed by the hot-plate method. Briefly, each mouse was injected s.c. with nicotine and tested 5 min after injection. Mice were placed on a hot plate (Thermostat Apparatus, Columbus, OH) maintained at 55˚C. Latency to reaction time (jumping or paw licking) was recorded. A control response (8-12 sec) was determined for each mouse before treatment and test latency was determined after drug administration. A maximum latency of 40 sec was imposed. Antinociceptive response was calculated as %MPE, where %MPE = [(test - control)/(40 - control) x 100].

Locomotor Activity. Mice were placed into individual Omnitech photocell activity cages (Columbus, OH; 28 x 16.5 cm) 10 min after s.c. administration of nicotine. Interruptions of the photocell beams (two banks of eight cells each) were then recorded for the next 10 min. Data are expressed as number of photocell interruptions.

Body Temperature. Rectal temperature was measured by a thermistor probe (inserted 24 mm) and digital thermometer (Yellow Springs Instrument Co., Yellow Springs, OH). Readings were taken just before and at 30 min after the s.c. injection of nicotine. The difference in rectal temperature before and after treatment was calculated for each mouse. The ambient temperature of the laboratory varied from 21-24°C from day to day.

Tolerance studies

Mice were implanted with Alzet osmotic mini-pumps (model 2002- Alza Corporation, Palo Alto, CA) filled with either (-)-nicotine (48 mg/kg/day) or sterile physiological saline solutions. The mini-pumps were surgically implanted s.c. under sterile conditions with pentobarbital anesthesia (35 mg/kg, i.p.). An incision was made in the back of the animals, and a pump was inserted. Animals were sutured and allowed to recover before being returned to their home cages. Mice were infused with nicotine or saline for 10 days and on day 11, they were challenged with different nicotine doses and tested for antinociception (tail-flick and hot-plate tests) and hypothermia. Calculations for MPE were performed as described previously.

Statistical Analysis

Statistical analysis of all behavioral studies was performed with mixed-factor ANOVA with post-hoc Tukey's test when appropriate. P-values of <0.05 were considered to be statistically significant. For chronic tolerance studies, ED_{50} (effective dose 50%) values were calculated with 95% confidence intervals by unweighted least-squares linear regression as described by Tallarida and Murray (1987). Tests for parallelism were calculated according to the method of Tallarida and Murray (1987). If confidence limit values did not overlap, then the shift in the dose-response curve was considered significant. Potency ratios were also calculated by dividing nicotine ED_{50} values by saline ED_{50} values for each age group and pharmacological measure to determine whether tolerance differences were significant between age groups.

C. Results

Nicotine's pharmacological effects after acute injection

Dose-response relationships were established for nicotine in male and female mice of both ages by measuring antinociception (two tests), hypothermia and hypomotility at the time of maximal effect (Fig. 10) and ED_{50}s (CL) values were then determined for each age in different tests (Table 1).

Males

In the tail-flick test, early adolescent male mice displayed decreased sensitivity as compared to male adult mice (Fig. 10a). ED_{50} values with confidence limits were 1.7 (1.3-3.6) and 1.0 (0.6-1.2) mg/kg for adolescent and adult age groups, respectively (Table 1). However, no significant differences between the two age groups were observed in the hot-plate, hypothermia, and hypomotility tests (Fig. 10b, 10c, 10d). In addition, all acute responses to nicotine in adult and adolescent mice were blocked by mecamylamine at 1.0 mg/kg (data not shown). Baseline levels in the tail-flick and hot-plate tests were not significantly different between the two ages, respectively (PND 21: 2.9 ± 0.2, 12.5 ± 1.3, PND 70: 2.7 ± 0.3, 13.0 ± 1.8 sec).

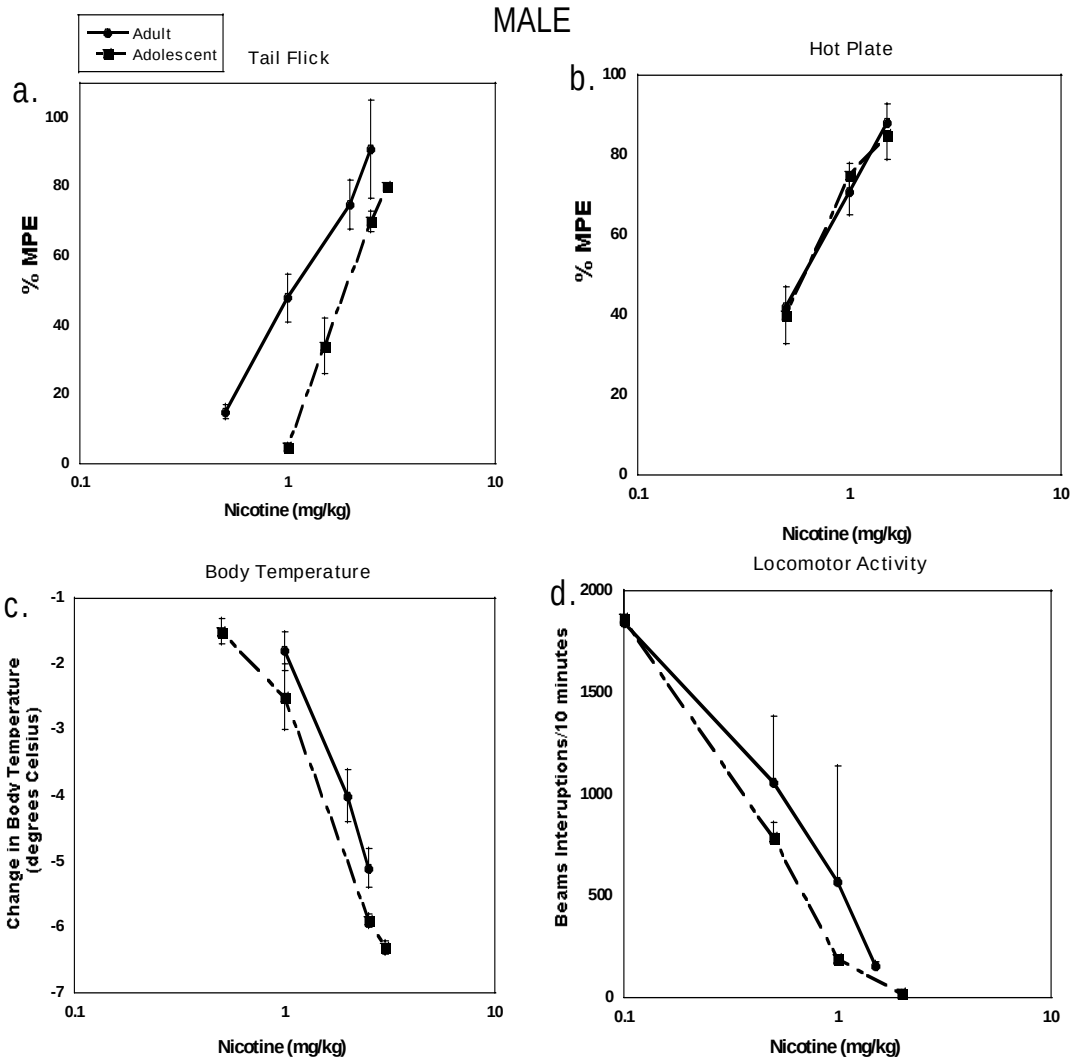

Figure 10. Nicotine's acute pharmacological effects in adult and adolescent male mice. Mice from two age groups (PND 21 and PND 70) were injected s.c. with various acute doses of nicotine and tested in the following responses: (a) tail-flick test, (b) hot-plate test, (c) hypothermia, and (d) locomotor activity. Each point represents the mean ± S.E. of 12 mice.

Females

Females also showed no differences in baseline measures for tail-flick and hot-plate tests: (PND 28: 2.5 ± 0.2, 12.2 ± 0.5, PND 70: 2.4 ± 0.2, 13.8 ± 1.0). The hot-plate and hypothermia measures revealed that early adolescent mice displayed increased sensitivity as compared to adult mice (Fig. 11b and 11c). In the hot-plate test, ED_{50} values with confidence limits were 0.4mg/kg (0.3-0.6mg/kg) and 0.9mg/kg (0.8-1.2mg/kg) for adolescent and adult age groups respectively (Table 1). Likewise, ED_{50} values in the hypothermia measure were 0.5mg/kg (0.2-0.8mg/kg) and 1.3mg/kg (0.9-2.0mg/kg) for adolescent and adult mice respectively. In addition, all acute responses to nicotine in adult and adolescent mice were blocked by mecamylamine at 1.0 mg/kg (data not shown).

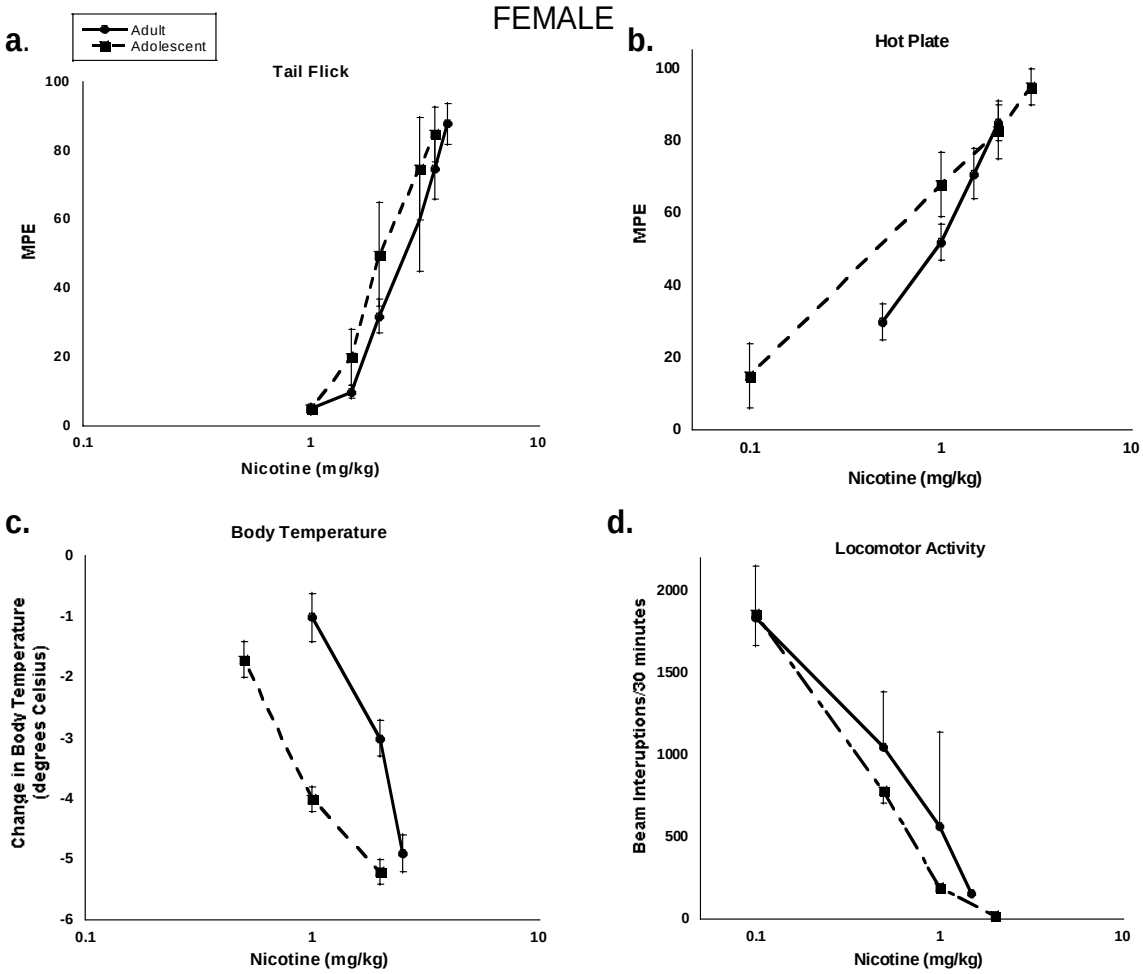

Figure 11. Nicotine's acute pharmacological effects in adult and adolescent female mice. Mice from two age groups (PND 21 and PND 70) were injected s.c. with various acute doses of nicotine and tested in the following responses: (a) tail-flick test, (b) hot-plate test, (c) hypothermia, and (d) locomotor activity. Each point represents the mean ± S.E. of 12 mice.

	PND 21 Male	PND 70 Male	PND 21 Female	PND 70 Female
Tail-flick	1.7 (1.3-3.6)*	1 (0.6-1.2)	2.3 (0.7-4.5)	3 (1.8-5.6)
Hot-plate	0.5 (0.4-0.6)	0.5 (0.4-0.6)	0.4 (0.3-0.6)*	0.9 (0.8-1.2)
Hypothermia	0.7 (0.5-1.9)	1.1 (0.5-2.2)	0.5 (0.2-0.8)*	1.3 (0.9-2.0)
Hypomotility	0.5 (0.15-2)	0.3 (0.1-0.8)	0.4 (0.1-2.4)	0.4(0.1-0.6)

Table 1: Summary of nicotine potency in young and adult mice after acute injections. Mice from two age groups (PND 21 and PND 70) were injected s.c. with various doses of nicotine and tested in the following responses: tail-flick test; hot plate test; hypothermia; locomotor activity. ED_{50} values ± confidence limits (± CL) were calculated from the dose-response curve of the respective treatment and expressed as mg/kg. Each dose group included 12 animals. * Indicates significant age differences as compared to adult (CLs do not overlap).

Development of tolerance to nicotine after chronic exposure

Males

As shown in Fig. 12, tolerance developed to nicotine's antinociceptive and hypothermic effects in both adult and adolescent male mice as reflected by the rightward shift in the dose-response curves. Furthermore, these shifts were significant as demonstrated by the significant increase (with non-overlapping confidence limits) in ED_{50} values after chronic nicotine (Table 2). To determine if the degree of tolerance was significantly different between the age groups, we calculated potency ratios for each group (Table 4). Adolescent male mice showed a higher degree of tolerance in the hot-plate test [potency ratios with confidence limits for adolescent and adults are 2.31 (2.03-2.62) and 1.75 (1.47-1.98)] respectively; however, tolerance to the tail flick and body temperature developed at the same degree.

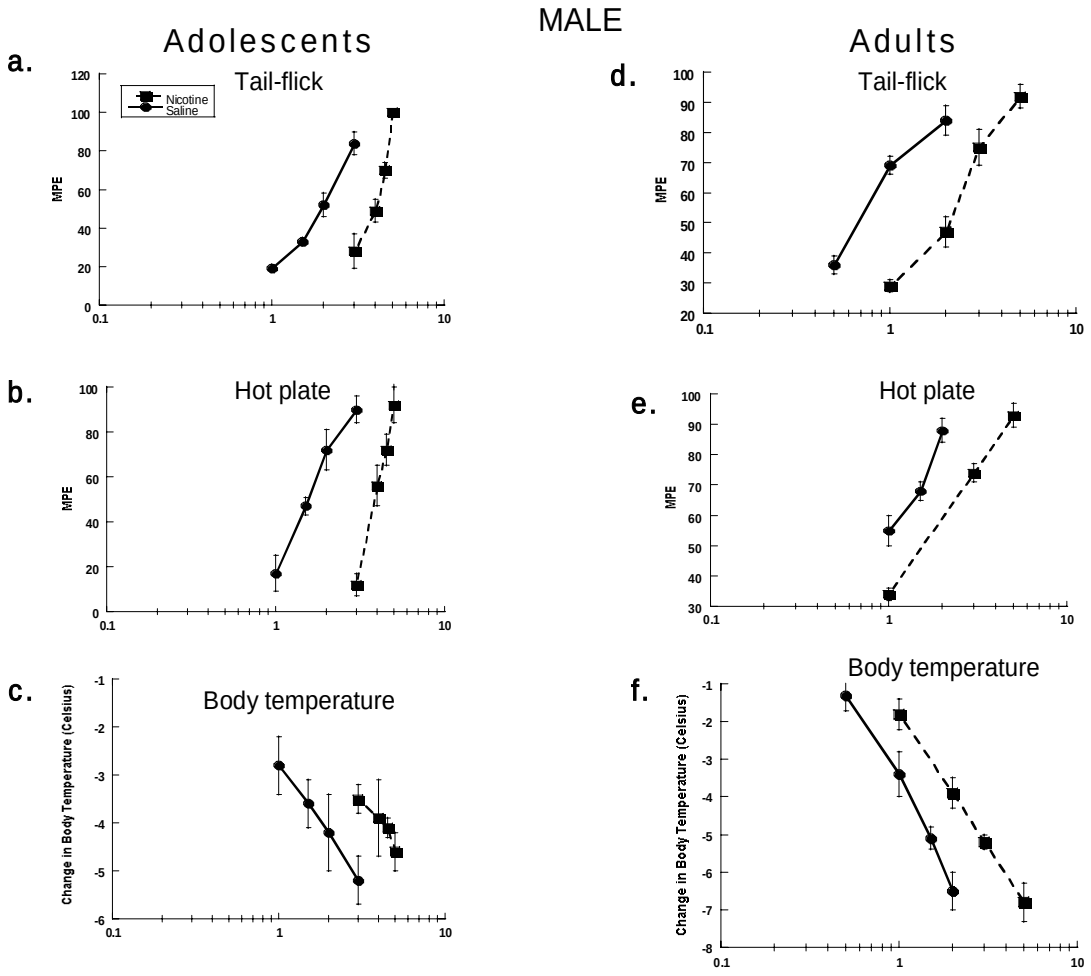

Figure 12. Dose-response curve of nicotine after chronic administration in adult (PND 70) and adolescent (PND 21) male mice. Animals were chronically infused with saline or nicotine at 48 mg/kg/day for 10 days via osmotic mini-pump. On day 11 mice were challenged with nicotine and evaluated in tail flick and hot plate analgesia and hypothermia. Adolescent mice are shown in graphs (a), (b), and (c) and adult mice are shown in graphs (d), (e), and (f). Each point represents the mean ± S.E. of 12 mice.

	Adolescent Saline	Adolescent Nicotine	Adult Saline	Adult Nicotine
Tail-Flick Test	1.8 (1.6-1.9)	3.7 (3.5-3.8) *	0.8 (0.7-0.9)	1.8 (1.5-2.0) *
Hot-Plate Test	1.5 (1.1-1.7)	3.8 (3.67-4.0) *	0.9 (0.8-1.0)	1.5 (1.3-1.7) *
Hypothermia	1.1 (0.8-1.5)	2.3 (1.8-3.0) *	0.9 (0.8-1.1)	1.4 (1.3-1.6) *

Table 2. ED_{50} values of tolerance studies after chronic administration of nicotine in adult and adolescent male mice. Mice were chronically infused with nicotine at 48 mg/kg/day for 10 days via osmotic mini-pump. On day 11 mice were challenged with nicotine and evaluated in three tests: tail-flick, hot-plate, and hypothermia. ED_{50} values ± Confidence limits (± CL) were calculated from the dose-response curve of the respective treatment and expressed as mg/kg. Each dose group included 12 animals. *Indicates a significant difference as compared to the saline control (CLs do not overlap).

Females

As shown in Fig. 13, tolerance also developed to nicotine's antinociceptive effects in female mice of both ages. These shifts were statistically significant as demonstrated by the increase in ED_{50} values after chronic nicotine (Table 3), which have non-overlapping confidence limits. Table 4 summarizes the potency ratios which are indicative of whether tolerance levels were significantly different between the early adolescents and adults. Indeed, female adolescent mice showed a lower degree of tolerance in the hypothermia measure [potency ratios with confidence limits for adolescent and adults are 1.35 (1.10-1.69) and 1.93 (1.77-2.23) respectively].

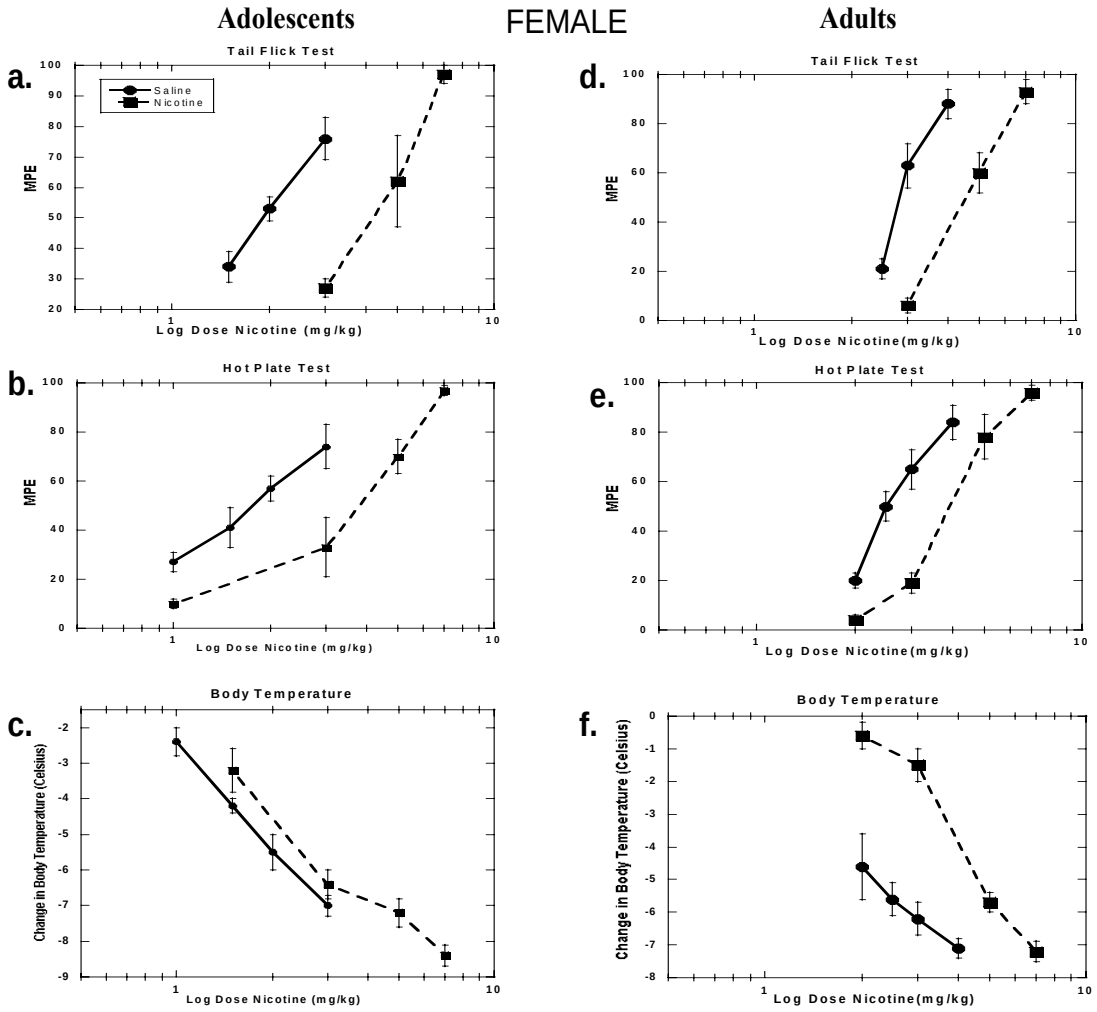

Figure 13. Dose-response curve of nicotine after chronic administration in adult (PND 70) and adolescent (PND 21) female mice. Animals were chronically infused with saline or nicotine at 48 mg/kg/day for 10 days via osmotic mini-pump. On day 11 mice were challenged with nicotine and evaluated in tail flick and hot plate analgesia and hypothermia. Adolescent mice are shown in graphs (a), (b), and (c) and adult mice are shown in graphs (d), (e), and (f). Each point represents the mean ± S.E. of 12 mice.

	Adolescent Saline	Adolescent Nicotine	Adult Saline	Adult Nicotine
Tail-Flick Test	1.9 (1.7-2.1)	3.2 (2.4-4.2)*	2.8 (2.7-3.0)	4.3 (3.9-4.8)*
Hot-Plate Test	1.7 (1.4-2.0)	3.0 (2.4-3.7)*	2.6 (2.4-2.8)	3.8 (3.5-4.8)*
Hypothermia	0.8 (0.6-1.1)	1.0 (0.9-1.2)	1.4 (1.1-1.7)	3.4 (3.1-3.7)*

Table 3. ED_{50} values of tolerance studies in female mice. Female ICR mice were chronically infused with nicotine at 48 mg/kg/day for 10 days via osmotic mini-pump. On day 11 mice were challenged with nicotine and evaluated in three tests: tail-flick, hot-plate, and hypothermia. ED_{50} values ± Confidence limits (± CL) were calculated from the dose-response curve of the respective treatment and expressed as mg/kg. Each dose group included 12 animals. *Indicates a significant difference as compared to the saline control (CLs do not overlap).

	Adolescent Males	Adult Males	Adolescent Females	Adult Females
Tail-Flick Test	1.9 (1.7-2.1)	2.0 (1.7-2.3)	1.5 (1.2-1.9)	1.4 (1.3-1.6)
Hot-Plate Test	2.3 (2.0-2.6)*	1.7 (1.4-1.9)	1.7 (1.3-2.2)	1.4 (1.3-1.6)
Hypothermia	2.2 (1.2-3.6)	1.6 (1.3-1.9)	1.3 (1.1-1.6)*	1.9 (1.7-2.2)

Table 4. Potency ratios for tolerance studies in male and female ICR mice. Potency ratios with confidence intervals are given for each group. * Indicates a significant difference between adolescent and adult groups in a particular test (confidence limits do not overlap).

D. Discussion

Acute sensitivity and tolerance to nicotine were examined in this chapter as possible underlying mechanisms of age-specific behavioral differences in components of nicotine dependence. Following acute treatment with nicotine, adolescent male mice displayed a nicotine-induced antinociception compared to adults in the tail-flick test. This finding suggests that, in male adolescents, predisposition to maintain use of nicotine might be due to the lessening of aversive effects due to decreased sensitivity to the drug. However, since there was only a decrease in the tail-flick test while the other measures showed no changes between the two age groups, it does not appear that acute sensitivity to nicotine is a major contributing factor.

The data from our tolerance study show that adolescent male mice produce a greater degree of tolerance to nicotine-induced antinociception as compared to adult mice in the hot plate test. The tolerance level is an important factor in evaluating nicotine dependence. The higher tolerance seen in male adolescents suggests that this age group would have to smoke more to achieve the same level of effect as an adult leading to a greater intake of nicotine in adolescence and an increase in the likelihood of becoming dependent. However, again only one measure of tolerance was significantly different between the two age groups in males suggesting a minor role for this mechanism.

Similarly in females, initial sensitivity to acute nicotine and tolerance to nicotine did not appear to play a large role in the differences in nicotine dependence between adults and adolescents. Adolescent female mice displayed an increased sensitivity to

acute nicotine treatment as compared to adults in the analgesic and hypothermia tests. This factor may contribute to the difficulty in quitting, but probably does not contribute greatly since not all measures were indicative of increased sensitivity. The data from our tolerance study show that female adolescent mice produce a lower degree of tolerance to nicotine-induced hypothermia as compared to adult mice, but no differences were observed in antinociceptive measures. Since we only saw differences in potency in one measure, it is also unlikely that this factor is a substantial contributor.

Taken together, these data suggest that differences in acute sensitivity and tolerance to nicotine only play a minor role in age-related differences in nicotine dependence. In addition to behavioral mechanisms, it is also important to consider variations in molecular and cellular mechanisms in order to understand drug dependence. In Chapter 5, we will consider mechanisms that examine receptor number and function, as well as differences in downstream signaling that may contribute to elevated levels of nicotine dependence in adolescents. Although differences in female mice were noted throughout *in vivo* studies, we have chosen to investigate the male sex for the remainder of the studies. We felt that it was beyond the scope of the project to address both sexes and have focused our attention on fully characterizing the male sex.

AN EXAMINATION OF THE PHASE OF ADOLESCENT NICOTINE EXPOSURE ON NICOTINE REWARD AND WITHDRAWAL IN MALES

A. Introduction

Chapters 2 and 3 demonstrate that early adolescent mice show different levels of nicotine dependence as compared to adult mice. However, it is important to know whether this specific phase of adolescence is unique or if age-related differences are also found during subsequent adolescent periods. Indeed, early adolescence has been implicated as the most critical period of adolescent development. Belluzzi et al. (2004) have shown that rats exposed to nicotine during early adolescence (P28) displayed conditioned place preference, while older adolescents and adults fail to do so. Moreover, Vastola et al. (2002) also found that only rats conditioned during early adolescence showed preference to nicotine. Behavioral studies in nicotine withdrawal have also focused on early adolescence (O'Dell et al. 2006; Shram et al. 2006) and have reached similar conclusions regarding age-specific differences. However, few studies have investigated other phases of adolescence to examine their susceptibility to dependence behaviors. One study reported that early adolescent mice demonstrated a preference for oral nicotine, while middle and late adolescents showed no preference (Adriani et al. 2002) suggesting the uniqueness of this phase. The aim of this chapter was to examine all three phases of adolescence in the CPP model and to examine the late adolescent phase in regards to signs of nicotine withdrawal. As a reminder, these

studies were only conducted in male subjects as studying these effects in females is beyond the scope of this project.

B. Methods

Nicotine-induced conditioned place preference studies

The same general procedure as previously described in Chapter 2 was used for this experiment. In addition to another set of early adolescent (PND 21) and adult (PND 70) mice, middle (PND 35) and late (PND 49) adolescent mice were also tested for nicotine-induced rewarding effects.

Precipitated nicotine withdrawal studies

Withdrawal testing was performed exactly as previously described in Chapter 2 using the osmotic mini-pump model. Mice were PND 49 upon mini-pump implantation and were tested 8 days later for mecamylamine-induced withdrawal signs.

Statistical Analysis

Statistical analysis of all behavioral studies was performed with mixed-factor ANOVA with post-hoc Tukey's test when appropriate. P-values of <0.05 were considered to be statistically significant.

C. Results

Effect of Adolescent Phase on Nicotine-Induced Conditioned Place Preference

Nicotine-induced CPP in all stages of adolescence is shown in Figure 14. Consistent with previous results in the adult, neither middle nor late adolescent mice displayed an enhanced CPP in response to low doses of nicotine. A significant preference was only established at a dose of 0.5 mg/kg for these three age groups. In

contrast, early adolescent mice demonstrated a clear preference for both 0.05 and 0.1 mg/kg nicotine which are inactive doses in older animals; thus suggesting an increased sensitivity to the rewarding effects of nicotine during this stage of development. These data support the hypothesis that early adolescence is the most critical stage for nicotine-induced rewarding effects.

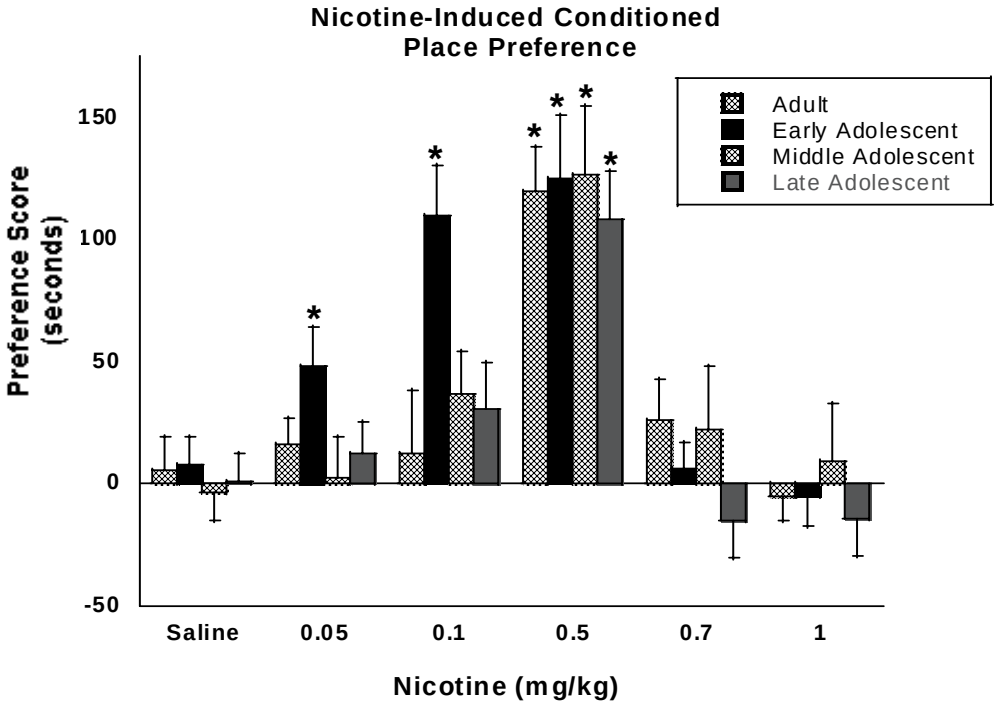

Figure 14. Age-dependent nicotine-induced conditioned place preference in male mice. Adult (PND 70), late adolescent (PND 49), middle adolescent (PND 35) and early adolescent (PND 21) were conditioned s.c. with various doses of either saline or nicotine using the CPP paradigm. Positive scores indicate a preference for nicotine while negative scores are indicative of aversion to the drug. Scores at or near zero indicate neither preference nor aversion. Each point represents the mean ± SEM of 8-9 mice. *p<0.05 from saline group.

Effect of Adolescent Phase on Nicotine Withdrawal

As with the conditioned place preference model, we also investigated how the stage of adolescence affected levels of nicotine withdrawal. We examined this question by precipitating nicotine withdrawal in late adolescent male mice after chronic administration of the drug. In order to easily compare adult, early adolescent and late adolescent data, we have graphed our results together as seen in Figure 15. Control groups were included in this experiment; however, no significant differences were noted so data is only shown in table format (Table 5) in order to simplify the graphs.

As previously stated, early adolescent mice displayed lower withdrawal signs as compared with adult mice. Particularly, somatic signs and hyperactivity measures were found to be significantly attenuated (Fig. 15a, 15d). Furthermore, there was no evidence of withdrawal behavior in the elevated plus maze (Fig. 15b) or in hyperalgesia (Fig. 15c) testing for early adolescent mice. On the other hand, late adolescent mice displayed withdrawal behavior in all four measures and there were no significant differences noted between adult and late adolescent withdrawal intensity. This suggests that early adolescents, but not late adolescents, have a decreased vulnerability to nicotine's aversive effects.

	Adult Sal MP/Sal Inj	Adult Nic MP/Sal Inj	Early Adolescent Sal MP/Sal Inj	Early Adolescent Nic MP/Sal Inj	Late Adolescent Sal MP/Sal Inj	Late Adolescent Nic MP/Sal Inj
SS	**4 ± 1**	**3 ± 1**	**3 ± 0**	**2 ± 0**	**2 ± 2**	**3 ± 1**
EPM	**28.5 ± 1.4**	**26.8 ± 3.0**	**33.6 ± 2.5**	**29.9 ± 1.8**	**30.7 ± 3.3**	**32.6 ± 2.0**
HP	**9 ± 1.1**	**10.6 ± 0.8**	**8.5 ± 0.3**	**7.5 ± 0.3**	**8.3 ± 0.9**	**8.6 ± 0.7**
LA	**1064 ± 98**	**968 ± 58**	**988 ± 65**	**1002 ± 42**	**1102 ± 54**	**1036 ± 39**

Table 5: Summary of control data for precipitated nicotine withdrawal experiments in adult (PND 70), early adolescent (PND 21), and late adolescent (PND 49) male mice. Mice were chronically infused with nicotine at 48 mg/kg/day or saline for 7 days. On day 8, mice were injected with 2.0 mg/kg of mecamylamine or saline s.c. to precipitate withdrawal and evaluated in four tests: somatic signs, elevated plus maze, hot plate analgesic test, and locomotor activity. MP=mini-pump; Inj=injection

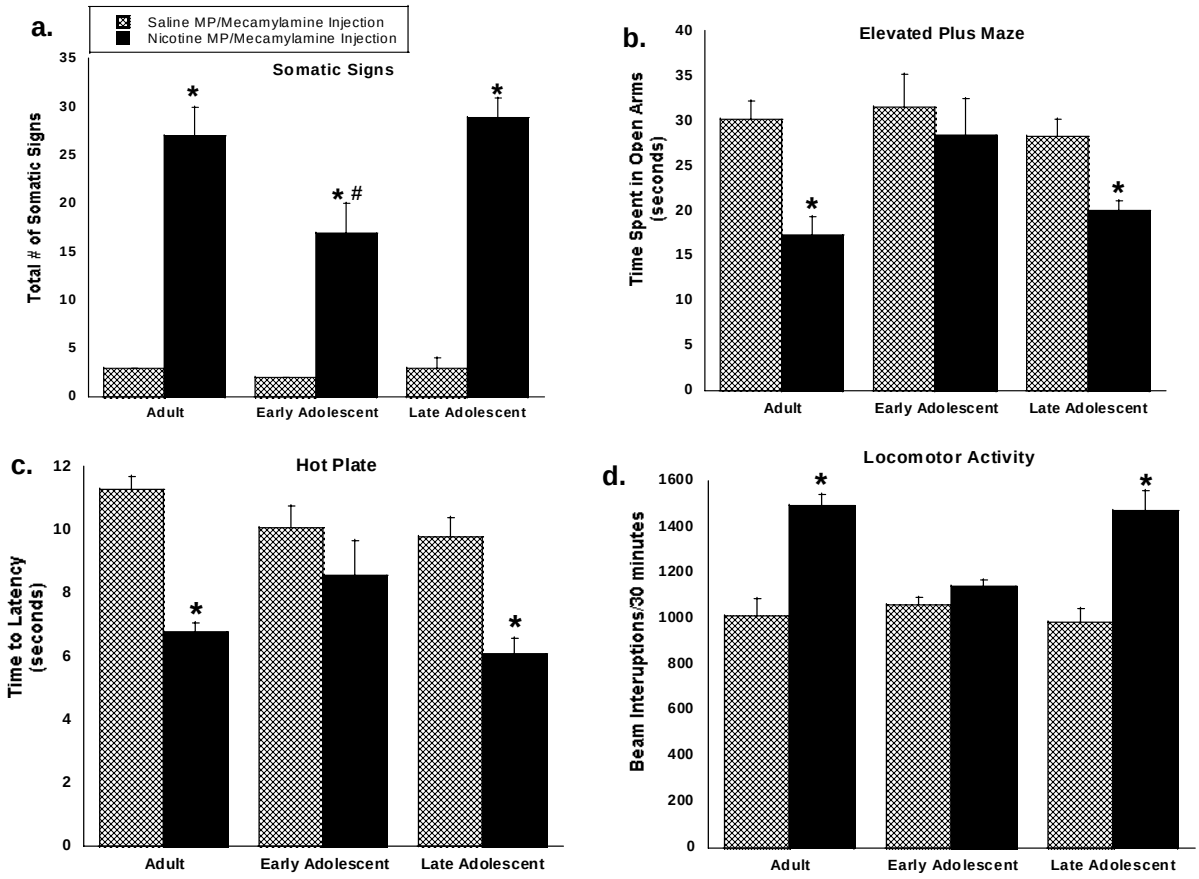

Figure 15. Mecamylamine-precipitated withdrawal in adult (PND 70), early adolescent (PND 21), and late adolescent (PND 49) male mice. Mice were chronically infused with nicotine at 48 mg/kg/day or saline for 7 days. On day 8, mice were injected with 2.0 mg/kg of mecamylamine or saline s.c. to precipitate withdrawal and evaluated in four tests: (a) somatic signs, (b) elevated plus maze, (c) hot plate analgesic test, and (d) locomotor activity. (n=12/group)

D. Discussion

Early adolescence has been identified as a critical period of both physical and neuronal development. Specifically, a vast amount of pruning and synapse loss has been reported. It has been estimated that as many of half of the average number of synapses are lost during adolescence (Rakic et al. 1994). These changes may contribute to differences in behavioral responses over certain periods of development. Only a limited number of studies have investigated nicotine's behavioral effects throughout all phases of adolescence. It is unclear if each phase is important in the development of drug dependence or if this susceptibility is limited to early adolescence. Data from our studies demonstrate that early adolescence is unique in both reward and withdrawal models.

Only early adolescent mice demonstrated a significant preference for low doses (0.05 and 0.1 mg/kg) of nicotine in the CPP model. This indicates that minimal exposure to nicotine may be able to elicit rewarding effects at this age due to enhanced sensitivity. In contrast, older adolescents and adults only displayed preference at a higher dose of nicotine: 0.5 mg/kg. Our data are in agreement with that of a previous study which found that only early adolescent mice displayed preference for oral nicotine (Adriani et al. 2002). In the withdrawal model, early adolescent mice demonstrated a significant attenuation of both physical and affective signs of withdrawal as compared to adults. On the other hand, late adolescents showed no differences from adults in any of the four withdrawal measures indicating that the intensity of withdrawal is similar in these two age groups.

Taken together, these data support the hypothesis that, in males, early adolescence represents a unique phase of development in which rewarding effects are enhanced and withdrawal signs are attenuated. This implies that humans may be more vulnerable to nicotine dependence if exposure begins at an early age. These findings stress the critical nature of early prevention messages and intervention strategies that combat teenage smoking. Indeed, studies have found that the commencement of smoking at a young age is thought to increase addiction, decrease the probability of successful cessation (Colby et al. 2000; Kandel and Chen 2000), and correlate with a higher number of cigarettes smoked per day (Taoli and Wynder 1991). It is important that we increase our understanding of the mechanisms behind this enhanced vulnerability so that smoking cessation therapies and treatments which are age appropriate can be properly developed. For these reasons, molecular studies will continue to focus on the early adolescent phase due to its apparent significance in the initiation of smoking.

MOLECULAR MECHANISMS INVOLVED IN NICOTINE DEPENDENCE

A. Introduction

Early adolescent and adult mice display different responses in models of nicotine reward and withdrawal. It is likely that functional properties, distribution, and number of nAChRs could contribute to these differences. Additionally, alterations in receptor number and function may lead to changes in downstream signaling which will affect pharmacological responses to nicotine. The goal of these experiments was to investigate these possibilities in order to further our understanding of early adolescent vulnerability to nicotine dependence.

Recently, it was reported that the quantity and distribution of nAChRs changes as development progresses. Specifically, Azam et al. (2007) found that α5, α6, and α7 mRNAs reach peak levels in early adolescence then decrease to lower adult levels. In addition, the authors reported regional differences in expression of α5, α6, and β3 mRNAs with elevated levels in the substantia nigra as compared to the ventral tegmental area. Furthermore, adolescent nicotine exposure appears to have important molecular consequences. One study examined the effects of acute nicotine exposure on several early response genes which are thought to be involved in synaptic plasticity and addiction. The study found that rats which were exposed to nicotine during adolescence showed an induction of arc mRNA in the PFC. Other genes, such as c-fos, were also upregulated by nicotine independent of age (Schochet et al. 2005). Finally, research by Levin et al. (2007) correlated an increase in nicotine i.v. self-administration in adolescent rats with significantly greater high affinity nicotinic receptor (α4β2) binding in the midbrain and the striatum as compared to adults. Taken together, these data suggest that adolescent nicotine exposure has important consequences on brain

maturation and development. Furthermore, nicotine may be causing alterations in receptor subtypes and function which will have critical downstream effects that may lead to increases in addiction vulnerability. Although some studies have examined the effect of nicotine on downstream signaling in adolescent rodents, only limited studies have directly compared age-related differences in distribution and expression of nAChRs before and after nicotine exposure.

Our approach in investigating these mechanisms involved beginning at the major target of nicotine, the nicotinic acetylcholine receptor. Rubidium efflux is a well-known and well-established model which is used to assess nAChR function. Many of the behavioral responses which have been examined in these studies are mediated by the α4β2* subtype (the major subtype measured in this assay) which is why we began with this approach. In addition to rubidium efflux, we performed nAChR binding studies to investigate basal differences in receptor levels between adults and early adolescents. Although this method is not a direct measure of receptor function, it has an advantage over assessing mRNA levels in that it directly measures protein levels. Finally, our dopamine release assay was performed in order to investigate particularly important effects downstream of the receptor activation. Dopamine is known to be an important neurotransmitter which is involved in the mesolimbic reward pathway that contributes to the addictive nature of nicotine. Together these studies aim to enhance the understanding of the molecular and cellular pathways of nicotine dependence.

B. Methods

Rubidium Efflux Studies

Unless otherwise noted, all reagents were purchased from Sigma Chemical Co., St. Louis, MO. Mice were rapidly decapitated and four brain regions were dissected for use in the assay (striatum, frontal cortex, hippocampus, and thalamus). Brain regions

were pooled from several mice if needed. In this experiment, we used striatum from 5 mice, cortex from 2 mice, hippocampus from 3 mice, and thalamus from 1 mouse. Synaptosomes were prepared according to Marks et al. (1993a, 1993b). Briefly, synaptosomes were prepared by hand homogenizing tissue in cold 0.32M sucrose (1ml/g tissue). After centrifugation, pellets were resuspended in cold load buffer (140mM NaCl, 1.5mM KCl, 2mM $CaCl_2$, 1mM $MgSO_4$, 25mM HEPES hemisodium salt, 20mM glucose, pH 7.4). A 25-µl aliquot of the synaptosome suspension was incubated for 40 min with 10µl load buffer containing approximately 4µCi $^{86}RbCl$ (Perkin Elmer Life Sciences, Boston, MA). After the synaptosomes were filtered onto glass fiber filters under gentle vacuum, the filters were rinsed with 0.5ml of load buffer and placed on the perfusion apparatus for washing with perfusion buffer (135mM NaCl, 5mM CsCl, 1mM $MgSO_4$, 2mM $CaCl_2$, 1.5mM KCl, 1g/l bovine serum albumin, 50nM tetrodotoxin, 25mM HEPES hemisodium salt, pH 7.4) for six min. The filter containing synaptosomes was subsequently perfused continuously. Filters were stimulated for one minute with various concentrations of nicotine prepared in perfusion buffer followed by a three-minute wash with perfusion buffer alone. Twelve-second fractions were collected in 12 x 75-mm test tubes beginning six min into the perfusion. Samples were counted for one minute each in a Wallac Wizard 3" 1480 Automatic Gamma Counter; (PerkinElmer, Shelton, CT). The magnitude of $^{86}Rb^+$ efflux response was calculated based on the increase in counts above baseline after stimulation of the tissue with nicotine. Data were calculated as fractional release (cpm/total cpm loaded on filter) for each fraction collected. The baseline was calculated for each mouse by fitting to an exponential equation the fractional release in fractions immediately preceding and following the peak. The area under the curve was calculated for each mouse using this mathematically derived baseline and the fractional release values in the peak.

Nicotinic Receptor Binding

Materials

(±)-[3H]Epibatidine (48 Ci/mmol) and L-[3H]nicotine (78 Ci/mmol), were purchased from Du Pont NEN (Boston, MA). alpha -[125I]Bungarotoxin (Initial specific activity = 220 Ci/mmol) plastic tritium standards and Hyperfilm-3H were purchased from Amersham (Mount Prospect, IL). NaCl, KCl, MgSO4, CaCl2, gelatin, chromium aluminum sulfate, cytisine, acetylcholine and diisopropyfluorophosphate were obtained from Sigma Chemical Co. (St. Louis, MO). Methylcarbachol chloride, (+)-epibatidine tartrate, and (-)-epibatidine tartrate were obtained from RBI (South Natick, MA). Nicotine bitartrate was a product of BDH Chemicals (Poole, England). Glass fiber filters Type A/E were obtained from Gelman Sciences (Ann Arbor, MI) and Type GB from MFS (Dublin, CA). Budget Solve scintillation fluid was obtained from RPI (Arlington Heights, IL).

Tissue preparation

Each mouse was killed by cervical dislocation; the brain was removed from the skull and placed on an ice-cold platform. The following 4 brain regions were dissected: nucleus accumbens (NAc), ventral tegmental area (VTA), hippocampus (HIP), and prefrontal cortex (PFC). Samples were homogenized in ice-cold hypotonic buffer (NaCl, 14.4 mM; KCl, 0.2 mM; CaCl2, 0.2 mM; MgSO4, 0.1 mM, HEPES, 2.0 mM; pH = 7.5) using a glass-Teflon tissue grinder. The particulate fraction was obtained by centrifugation at 20,000 × g for 20 min in a Sorvall RC-2B centrifuge. The pellet was resuspended in fresh homogenization buffer, incubated at 37°C for 10 min, and harvested by centrifugation. Each sample was washed twice more by resuspension and centrifugation and stored as a pellet under homogenization buffer at -70°C until use.

[^{3}H]nicotine binding

The binding of [^{3}H]nicotine was measured using a modification of the method of Marks et al. (1986). Samples (50-200 µg, depending on brain region) were incubated in 96-well polystyrene plates with 20 nM [^{3}H]nicotine at 22°C for 30 min in 100 µl of binding buffer (NaCl, 144 mM; KCl, 1.5 mM, CaCl2, 2 mM; MgSO4, 1 mM; HEPES, 20 mM; pH = 7.5). The binding reaction was terminated by filtration of the samples onto glass fiber filters (MFS GB top, Gelman A/E bottom) that had been soaked in binding buffer containing 0.5% polyethylenimine using an Inotech Cell Harvester (Inotech, East Lansing, MI). Samples were subsequently washed six times with ice-cold binding buffer. Nonspecific binding was determined by including 10 µM L-nicotine in the assay.

alpha -[^{125}I]bungarotoxin binding

The binding of alpha -[^{125}I]bungarotoxin was measured using a modification of the method of Marks et al. (1986). The binding reaction was similar to that used for [^{3}H]nicotine with the following changes: incubation time was 5 hr, samples contained 1 nM alpha -[1^{25}I]bungarotoxin instead of [^{3}H]nicotine and the binding buffer also included .025% bovine serum albumin. Blanks were determined by including 1 mM L-nicotine in the assay.

[^{3}H]epibatidine binding

The binding of [^{3}H]epibatidine was measured in a method analogous to that of [^{3}H]nicotine with the following changes: incubations were in 1-ml polypropylene tubes in a 96-well format, incubation volume was 500 µl, and [^{3}H]epibatidine rather than [^{3}H]nicotine was used. Nonspecific binding was determined by including 100 µM L-nicotine in the assay. Nonspecific binding at all concentrations of [^{3}H]epibatidine was less than twice background (40 dpm). The following experiments were conducted:

construction of curves for inhibition of [^{3}H]epibatidine binding in olfactory bulbs by cytisine, nicotine, acetylcholine (using tissue treated with 10 μM diisopropylflourophosphate during the tissue preparation), methylcarbachol, (+)-epibatidine and (-)-epibatidine (preliminary experiments indicated that inhibition in olfactory bulbs deviated markedly from that expected for a single site); construction of curves for inhibition of [^{3}H]epibatidine binding in 4 brain regions by cytisine; and measurement of the concentration dependence of [^{3}H]epibatidine binding in 4 brain regions. The concentration of [^{3}H]epibatidine used for inhibition curves was about 400 pM (approximately 20 x Kd). This concentration was chosen to maintain ligand binding to the tissue to less than 5% of the total. An incubation time of 60 min was used for these experiments (equilibrium was reached in 20-30 min). For saturation curves, eight [^{3}H]epibatidine concentrations between 6 and 800 pM were used. Incubation time for these experiments was 2 hr (equilibrium was reached by 60 min for all concentrations). In these experiments a significant fraction of the [^{3}H]epibatidine was bound to the tissue, especially at lower ligand concentrations. Free [^{3}H]epibatidine concentration was estimated by correcting for the amount of ligand bound to the tissue at each concentration for every brain region.

Protein

Protein was measured using the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Calculations

Results for saturation binding experiments were calculated using the Hill equation: $B = B_{max} * L^n / (L^n + K_d^n)$, where B is the binding at free ligand concentration, L, B_{max} is the maximum number of binding sites, K_d is the equilibrium dissociation constant, and n is the Hill coefficient. Values of B_{max}, K_d and n were calculated using

the nonlinear least squares algorithm in Sigma Plot 5 (Jandel Scientific, San Rafael, CA). Results for inhibition of epibatidine binding were calculated using the formulas for either one or two binding sites: $B = B_0/(1+(I/IC_{50}))$ or $B = B_1/(1+(I/IC_{50\text{-}1})) + B_2/(1+(I/IC_{50\text{-}2}))$, respectively, where B is ligand bound at inhibitor concentration, I, B_0 is the binding in the absence of inhibitor, and B_1 and B_2 are the binding to two sites sensitive to inhibition with $IC_{50\text{-}1}$ and $IC_{50\text{-}2}$. Assuming competitive inhibition: $IC_{50} = K_i \times (1 + L/K_d)$. Results were also calculated using the Hill equation.

Dopamine Release Assay

Materials

7,8-[^{3}H]Dopamine was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA) (specific activity, 40–60 Ci/mmol).

Membrane Preparation

Adult and adolescent male mice were sacrificed by cervical dislocation. The brain was removed from the skull and was immediately placed on ice for dissection. Striatum was isolated and removed from the brain. Tissue was homogenized (16-20 strokes by hand) in 0.5 ml of 0.32 M sucrose buffered with 5 mM HEPES, pH 7.5. Synaptosomal pellets were then prepared by centrifugation at 1000g for 10 min, followed by centrifugation of the resulting supernatant at 12,000g for 20 min. The pellets were resuspended in perfusion buffer (128 mM NaCl, 2.4 mM KCl, 3.2 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 25 mM HEPES, pH 7.5, 10 mM glucose, 1 mM ascorbic acid, and 0.01mM pargyline. The perfusion procedure has been described previously (Grady et al., 1997). Briefly, synaptosomes were incubated at 37°C in perfusion buffer for 10 min before addition of 100 nM [^{3}H]dopamine (1 µCi for every 0.2 ml of synaptosomes). Aliquots of synaptosomes (80 µl) were distributed onto filters and perfused at 0.6 ml/min for 10 min before fractions were collected. [^{3}H]dopamine

were added at the same time during the last 5 min of the uptake procedure. Atropine (1 μM) was added to the perfusion buffer to inhibit muscarinic receptors. Various concentrations of nicotine were used to stimulate dopamine release. Fractions were collected every 30s, and radioactivity was determined by scintillation counting (1600TR liquid scintillation spectrometer; Packard Instrument Co.) after addition of EconoSafe (Sigma/RBI, Mt. Prospect, IL).

Statistical Analysis

Nicotine stimulated $^{86}Rb+$ efflux was analyzed with a two-way analysis of variance (ANOVA) and a one-way ANOVA as a function of age. These were followed by Tukey post hoc tests. EC_{50} (effective concentration 50%) were calculated by unweighted least-squares linear regression as described by Tallarida and Murray (1987). A P value of <0.05 was considered statistically significant. nAChR binding studies were analyzed using two-way ANOVAs with appropriate post-hoc tests when necessary. EC_{50}s were calculated for dopamine release assay curves. Individual concentrations were also analyzed with two-way ANOVAs with Tukey post-hoc tests when appropriate.

C. Results

Rubidium Efflux Studies

Dose-response curves for nicotine-stimulated synaptosomes were generated for both adults and adolescents in four brain regions: striatum, cortex, hippocampus, and thalamus (Fig. 16; data represented by a Michaelis-Menten curve fit where y=m1*x/(m2+x)). Synaptosomes were responsive to nicotine stimulation in a dose-dependent manner until approximately 100μM of nicotine at which point the response

reached its peak. Adolescent mice displayed greater nAChR functionality with larger differences at lower concentrations of nicotine. The difference is also evident by the shift to the left of nicotine dose-response curves in adolescent compared to that of adult mice (Fig.16). Estimated EC_{50} values for both age groups are as follows in adolescents: striatum=0.53μM; cortex=0.79μM; hippocampus=0.96μM; and thalamus=0.53μM. EC_{50} values for adults were: striatum=1.61μM; cortex=10.77μM; hippocampus=3.13μM; and thalamus=3.93μM. We have coupled this analysis with the total AUC which is a more comprehensive measure of nAChR functionality. As shown in Fig. 17, adolescent mice displayed significantly higher nicotinic receptor functionality than adults in all four brain regions tested.

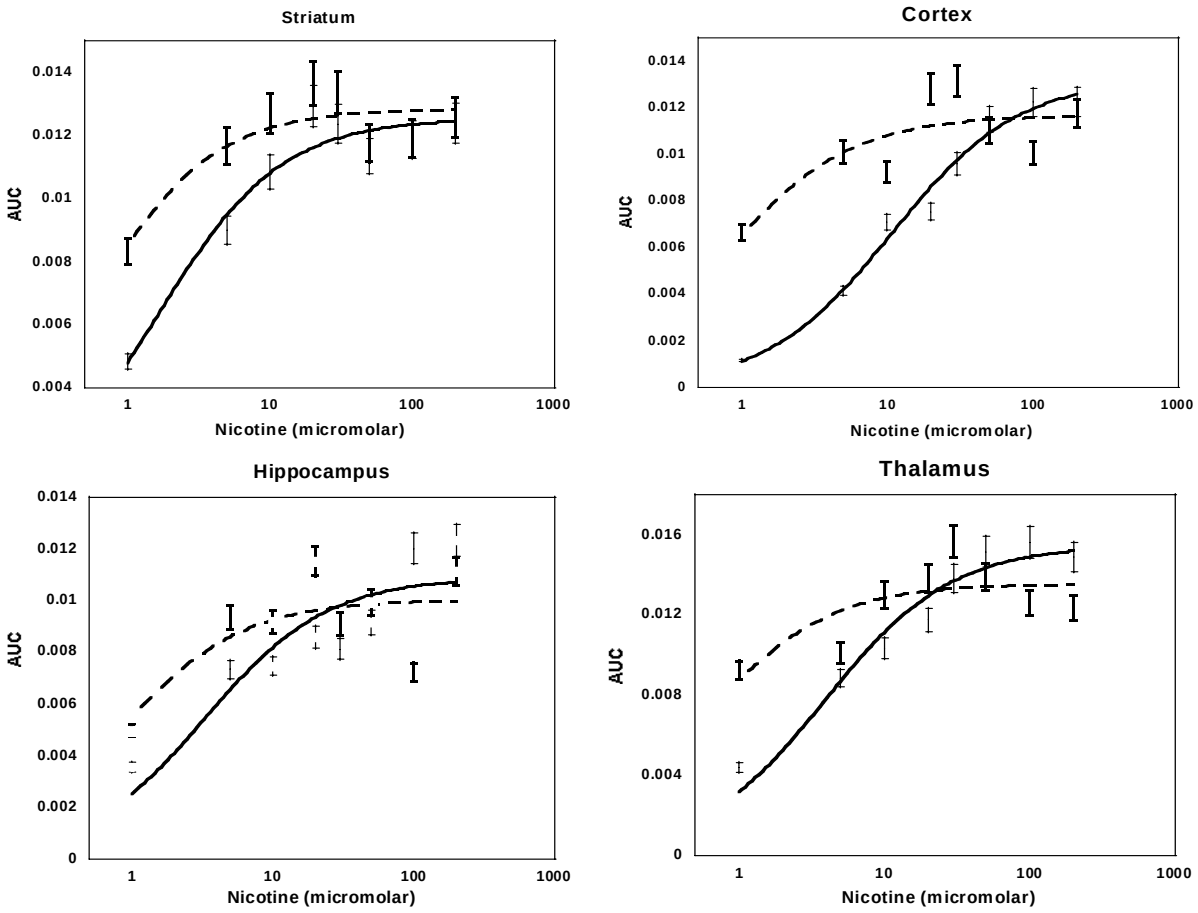

Figure 16. Dose response curves from the striatum, cortex, hippocampus, and thalamus regions of adult (PND 75) and adolescent (PND 28) male mice. Synaptosomes from brain tissue were stimulated with various doses of nicotine for one minute to generate dose-response curves. Area under the curve is shown on the y-axis and nicotine dose is shown on the x-axis. Data are represented by a Michaelis-Menten curve fit where y=m1*x/(m2+x). In the striatum, R values =0.97 (adolescents) and 0.87 (adults). In the cortex, R values = 0.99 (adolescents) and 0.81 (adults). Adolescent mice (dashed line) displayed higher nAChR functionality as compared to adult mice (solid line). Results are expressed as mean AUC ± S.E.

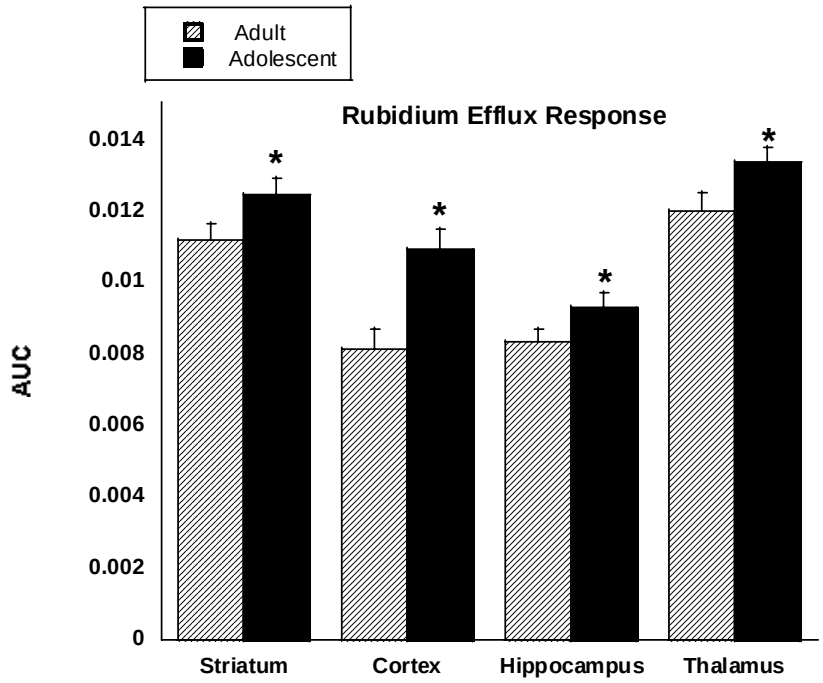

Figure 17. Total area under the curve for all doses of nicotine in the rubidium efflux assay in four brain regions. Results are expressed as mean AUC ± S.E. * p<0.05 from adult mice.

Nicotinic Receptor Binding Studies

Results from the nicotinic receptor binding studies are shown in Figure 18. Binding techniques quantified total nAChRs (a), cytisine sensitive α4β2* nAChRs (b), α-conotoxin-MII sensitive α6-containing nAChRs (c), and α-bungarotoxin sensitive α7 nAChRs (d) using various pharmacological tools. In the α4β2* and the total nAChR binding studies, a trend for increased nAChR binding in the adolescent mice was observed; however no significant differences were found in total nAChR binding or for a particular nAChR subtype.

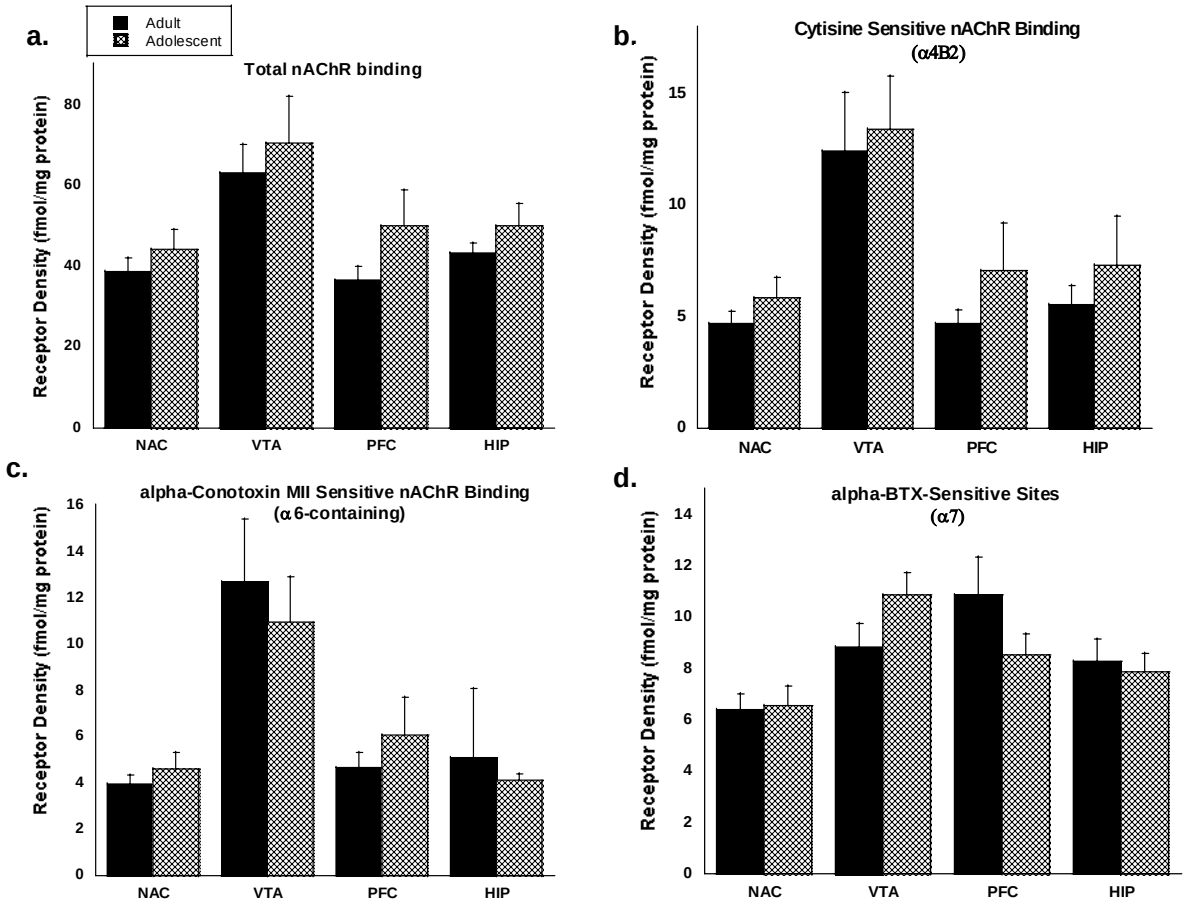

Figure 18. Nicotinic acetylcholine receptor binding assays were performed on adult (PND 75) and adolescent (PND 28) ICR mice. Various pharmacological tools were used to assess the following nAChR subtypes: (a) total nAChR binding; (b) cytisine-sensitive binding (α4β2); (c) alpha-conotoxin (α6); and (d) alpha-bungarotoxin (α7). Results are expressed as receptor density normalized to protein. Bars represent the mean ± S.E. of 6-8 mice.

Dopamine Release Assay

Figure 19 shows the results from the dopamine release assay which examined levels of dopamine release from the striatum in adult and adolescent mice. Dose-response curves were calculated for both age groups over a range of nicotine doses. The graph shows that there was a trend for an increased dopamine release from adolescent synaptosomes. However, this increase was not significant when all data was compiled together as total area under the curve.

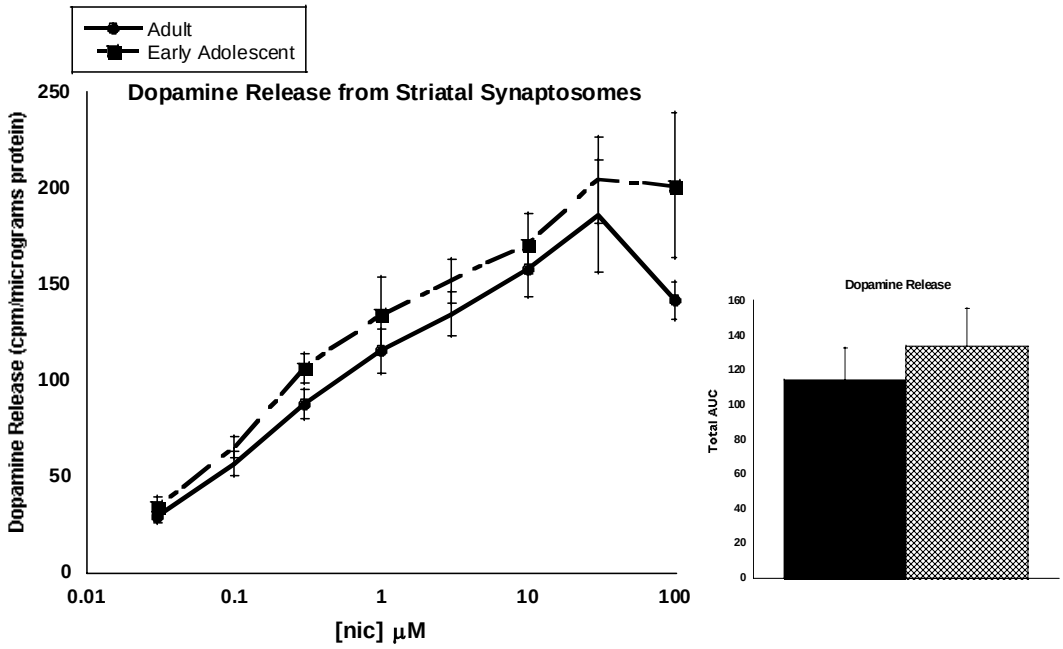

Figure 19. Dopamine release from striatal synaptosomes in adult (PND 75) and adolescent (PND 28) mice. Striatal synaptosomes were stimulated with various concentrations of nicotine to generate a dose-response curve. Each point represents the mean ± S.E. of 6-8 mice. Total area under the curve is also presented in the graph to the right with adults represented by the solid black bar and adolescents represented by the hatched bar. * p<.05 from same concentration in adult.

D. Discussion

The data in this chapter show that there is a clear and significant increase in nAChR functional response to nicotine in adolescent mice as compared to adults (Figures 16 and 17). This increase is consistent in four important brain regions (striatum, cortex, hippocampus, and thalamus) which contribute to nicotine addiction and dependence. Our data also agree with recent findings in the rat (Britton et al. 2007) where nicotine-stimulated rubidium efflux peaked during adolescence (~PND 35). This increased functionality of adolescent receptors could be playing a role in the behavioral observations seen in the conditioned place preference and withdrawal models. Our results support the observation of an enhanced preference in the CPP model in that increased nAChR function would translate into increased responsiveness to the rewarding effects of nicotine. In contrast, correlations to the results from our withdrawal studies are not as clear. There are differences between the two studies which may account for differences in the results. Rubidium efflux studies were conducted in naïve mice while withdrawal studies were conducted after chronic exposure to nicotine. It is possible that nAChRs may be regulated differently after chronic drug treatment which would contribute to inconsistencies.

These results could imply several other possibilities for age-related differences in behavioral models. It is logical to consider that increased functional response may be due an increase in basal levels of nAChRs in adolescent as compared to adult animals. For this reason, we investigated nAChR binding in the brain in these two ages. We found no significant differences in receptor binding in our study. Furthermore, specific

receptor subtypes were also evaluated and still no differences were observed (Figure 18). In contrast to our findings, Azam et al. (2007) reported that levels of α5, α6, and α7 mRNA were higher during the adolescent period. However, mRNA data must be interpreted with caution in that differences in mRNA do not necessarily reflect a similar change in receptor protein expression. Another possibility is that differences in receptor stoichiometry (i.e. $(\alpha4)_2(\beta2)_3$ vs. α4α5β2, etc) are not detectable by binding methods.

Since neuronal pathways are still developing in young animals, it is also possible that the adaptations in the brain during development cause the levels of dependence to change over time as well. For example, the dopaminergic system is under great development during adolescence and may account for behavioral observations. However, results from our dopamine release studies did not find significant differences between the two age groups in the mouse (Figure 19). On the other hand, Azam et al. (2007) reported that nicotine-stimulated dopamine release was significantly higher during the early adolescent period in the male rat. Furthermore, previous work has demonstrated that dopamine release is attenuated in the adult rat during withdrawal (DiChiara 2000; Hildebrand et al. 1998). Therefore, it is possible that adolescents do not experience this same decrease in dopamine thus lowering withdrawal symptoms and aversive effects. The study by Azam et al. (2007) was different from our study in that they were comparing nicotine-evoked dopamine release in animals at even younger ages (PND 7 and PND 14); whereas our study used PND 24-28 mice. In addition, no comparisons to adult rodents are given in this report. As previously mentioned, our assays which used a crude preparation of synaptosomes may

not ensure complete precision. For example, techniques such as microdialysis may be more accurate in that it preserves the neuronal connections of an intact neuron. It is possible that more sensitive assays may be required to denote differences between the age groups.

An alternative explanation for the behavioral responses which we have observed may be linked to other receptor types which are known to be involved in nicotine dependence. For example, glutamatergic receptors have been shown to play a role in nicotinic effects as well. Research has shown that administration of mGlu2/3 agonists decreased nicotine, but not food self-administration in rats (Liechti et al. 2007). Another study showed that nicotine exposure during adolescence dose-dependently down-regulated GluR2/3 subunits in the striatum and hippocampus while nicotine exposure in adults did not have this effect (Adriani et al. 2004). This same study also showed changes in NMDA NR2A/B subunits regardless of the time of exposure suggesting the involvement of NMDA receptors in certain aspects of nicotine dependence. These findings suggest that other receptors may also be involved and should be further examined.

"BEHAVIORAL PLASTICITY": THE EFFECTS OF ADOLESCENT NICOTINE EXPOSURE ON NICOTINE DEPENDENCE

A. Introduction

Overall, studies conducted to date suggest that the rewarding and reinforcing effects of nicotine are enhanced in adolescent versus adult rodents and that early adolescence may represent a period of heightened vulnerability. Data discussed in Chapter 2 indicate that in male adolescent mice, there is an increase in sensitivity to nicotine reward as well as attenuation in withdrawal signs as compared to adults. While it is clear that there are substantial behavioral age differences in nicotine dependence, it is still uncertain what type of long-lasting effects adolescent nicotine exposure has on lifetime nicotine dependence. Human studies have sought to examine this question since statistics support the concept that those who begin smoking at an early age are more likely to continue this pattern of behavior. Indeed, over 90% of adult smokers report their first use of tobacco prior to age 18 (Chassin et al. 1990). The commencement of smoking at a young age is thought to increase addiction, decrease the probability of successful cessation (Colby et al. 2000; Kandel and Chen 2000), and correlate with a higher number of cigarettes smoked per day (Taoli and Wynder 1991). Since more than 6,000 teenagers begin smoking every day (American Lung Association Statistics 2002) this is a critical problem which needs to be investigated. Human studies are limited in that they are unable to discern biological factors since many social, psychological, and emotional factors may also play an important role. For this reason, animal models are useful in that they have a biological emphasis.

The goal of this study was to conduct a thorough investigation of the effects of adolescent nicotine exposure on both nicotine reward and withdrawal in adulthood. Specifically, dose and duration of exposure were examined to determine how these two factors contribute to the induction of persistent behavioral changes. In addition to nicotine reward and withdrawal, locomotor function was measured following adolescent nicotine exposure. Finally, we investigated the correlation of adolescent nicotine exposure with measures of nAChR function using the rubidium efflux assay in order to assess whether early exposure had long-lasting effects at the receptor level. We hypothesized that chronic exposure to nicotine during early adolescence would have long-lasting effects on behavior in adulthood.

B. Methods

Drugs

(-)-Nicotine bitartrate and mecamylamine hydrochloride were purchased from Sigma Chemical Company (Milwaukee, WI). All doses are expressed as free base.

Conditioned Place Preference Studies

Mice received nicotine for various durations during early adolescence (PND 21-31), late adolescence (PND 49-59) or adulthood (PND 70-80). Table 6 fully describes the various experimental groups and the time course of this experiment. Briefly, there were three durations of nicotine exposure: acute, intermittent, and frequent. Two doses of nicotine (0.1 and 0.5 mg/kg) or saline were administered s.c. twice daily with injections being approximately 6 hours apart (9am and 3pm). Once adolescent mice had reached adulthood (PND 70), mice were evaluated for nicotine reward using

conditioned place preference. Mice which received their first injections in adulthood (PND 70) were evaluated seven weeks later (PND 120) in order to mimic the amount of time between early adolescence and adulthood.

The precise protocol for conditioned place preference was the same as previously described in Chapter 2. Briefly, mice have a pre-conditioning day which is a drug free assessment of baseline preference in a three compartment chamber. This is followed by three days of conditioning to either nicotine. Only one conditioning dose of nicotine was used in this model (nicotine 0.5 mg/kg). This dose was chosen because it was found to elicit preference for early adolescents and adults as demonstrated in Figure 2 of Chapter 2. The final day of the paradigm is the same as day 1 and assesses preference after the conditioning period. Preference scores are expressed as time spent on drug-paired side minus time spent on saline-paired side. A positive number indicated a preference for the drug-paired side, while a negative number indicated an aversion to the drug-paired side. A number at or near zero indicated no preference for either side.

Early adolescence-Acute	One day; PND 28; 2 total injections
Early adolescence-Intermittent	Every 3 days; PND 22, 25, 28, 31; 8 total injections
Early adolescence-Frequent	Every day; PND 22-28; 14 total injections
Late adolescence-Frequent	Every day; PND 50-56; 14 total injections
Adulthood-Frequent	Every day; PND 71-77; 14 total injections

Table 6. Time-course for conditioned place preference studies.

Mecamylamine-Precipitated Withdrawal Studies

The timeline of nicotine withdrawal studies are outlined in Figure 20. Naïve male mice were implanted with Alzet osmotic mini-pumps (model 2002- Alza Corporation, Palo Alto, CA) filled with either (-)-nicotine (48 mg/kg/day or 24 mg/kg/day) or sterile physiological saline solutions. The mini-pumps were surgically implanted s.c. under sterile conditions with pentobarbital anesthesia (35 mg/kg, i.p.). An incision was made in the back of the animals, and a pump was inserted. Animals were sutured and allowed to recover before being returned to their home cages. Eight days following each mini-pump implantation, mice were injected s.c with 2.0mg/kg of mecamylamine, a non-specific nicotinic antagonist, to precipitate withdrawal. Withdrawal testing was conducted as previously described in Chapter 2. Briefly, mice were assessed for withdrawal signs in a battery of four tests: 5 min for anxiety-like behavior (on the elevated plus maze), 20 min observation of somatic signs (paw tremors, head shakes, backing, body tremors, ptosis), hyperalgesia, and 30 min in locomotor activity chambers.

One day following withdrawal testing, mice were lightly anesthetized using ether and mini-pumps were removed. A small incision was made on the back of the neck in order to remove the mini-pump and the wound was closed with a suture. Mice were returned to their home cages in between surgeries and monitored on a weekly basis. Each experimental group was implanted with a second mini-pump according to

the timeline below (Figure 20). Withdrawal testing was conducted in the same manner as previously described.

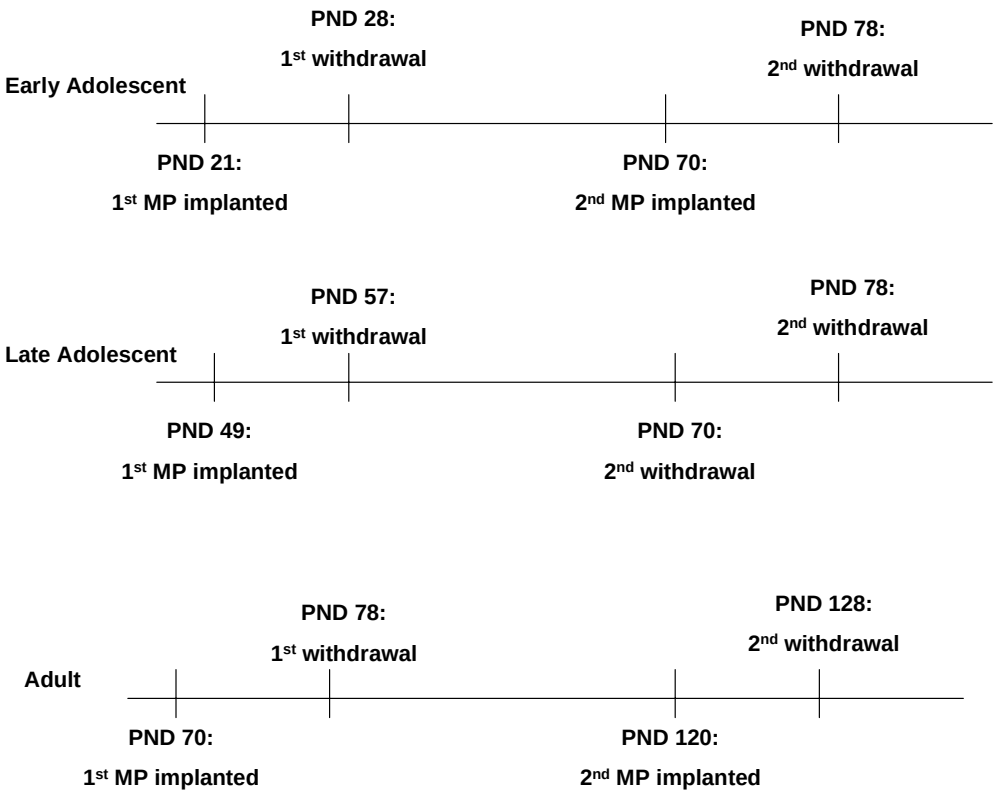

Figure 20. Timeline for nicotine withdrawal studies. Nicotine mini-pumps were implanted during early adolescence (PND 21), late adolescence (PND 49), or adulthood (PND 70) for 7 days. Withdrawal testing was precipitated by mecamylamine. After a recovery period, a second mini-pump was implanted and withdrawal testing was conducted again in the same manner. MP=mini-pump, PND = postnatal day

Rubidium Efflux Studies

Both early adolescent and adult male mice were injected s.c. with nicotine or saline for 7 days. Only one dose of nicotine (0.5 mg/kg) was used in these studies based on previous behavioral results indicating that this dose was effective at inducing long-term behavioral changes. The precise procedure for the rubidium efflux assay was the same as previously described in Chapter 5 except that only two concentrations of nicotine were utilized to stimulate the synaptosomes (1μM and 10μM).

C. Results

Effects of Adolescent Nicotine Exposure on Nicotine-Induced Rewarding Effects

Figure 21 presents the effects of early adolescent nicotine exposure on nicotine reward in adulthood. As expected, when conditioned with saline in the CPP paradigm, no preference was obtained. The nicotine challenge dose (0.5 mg/kg) did elicit a preference for the drug-paired side after all patterns of exposure. In panels (a) and (b), nicotine pretreatment in adolescence did not affect nicotine-induced reward in adulthood. On the other hand, panel (c) demonstrates that at the moderate dose of 0.5 mg/kg nicotine, repeated nicotine exposure during early adolescence does enhance nicotine reward in adulthood.

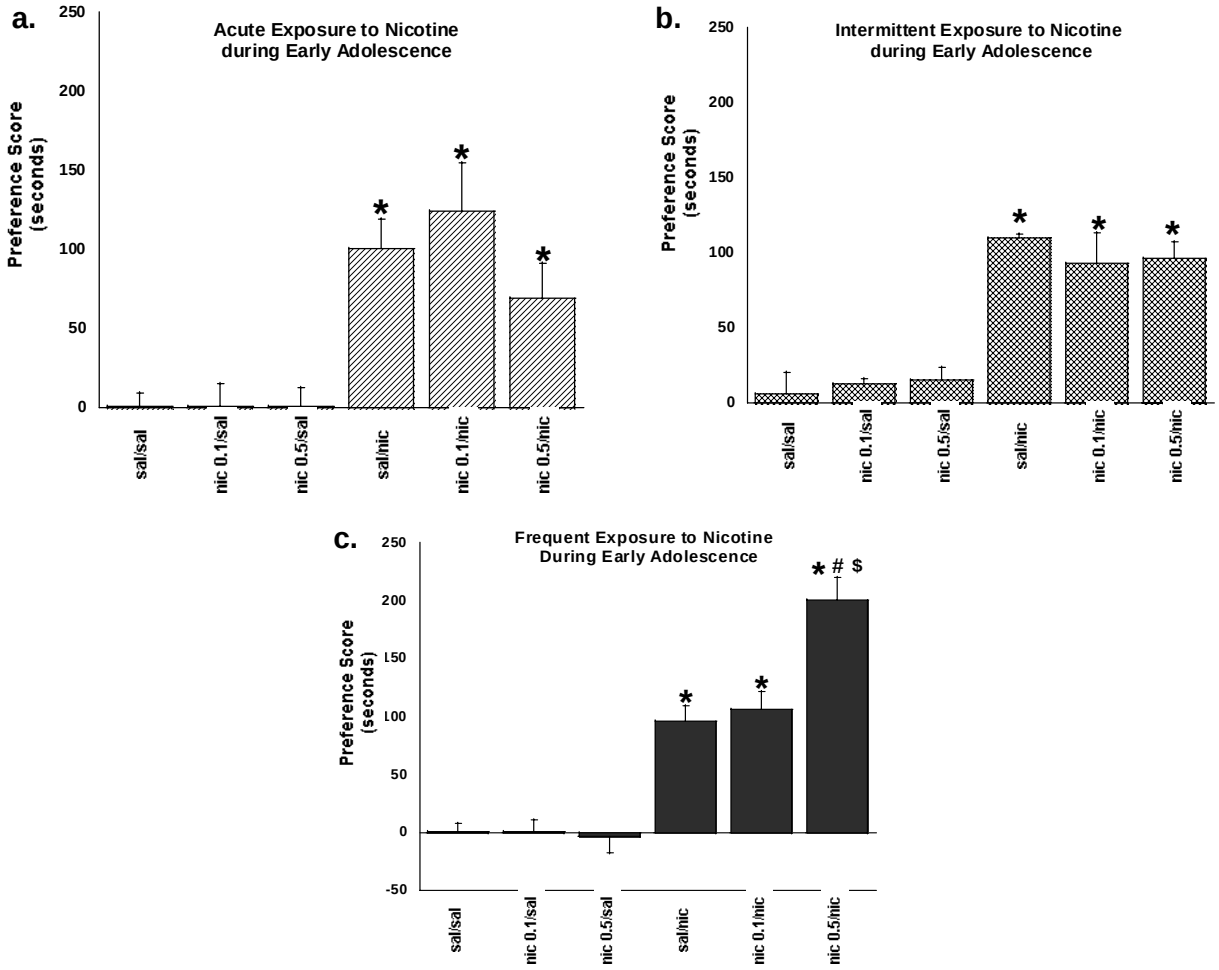

Figure 21. Effect of early adolescent nicotine exposure on nicotine-induced reward in adulthood. The y-axis represents preference score and the x-axis expresses adolescent treatment followed by treatment in the CPP paradigm. Short term (a) and intermittent (b) exposure to nicotine during early adolescence does not enhance nicotine-induced conditioned place preference in adulthood; however frequent exposure (c) to a moderate dose of nicotine during early adolescence results in elevated nicotine-induced reward through a CPP model. * p<.05 from respective saline control; # p<.05 from sal/nic group in the same graph; $ p<.05 from nic0.5/nic groups in acute (a) and intermittent (b) graphs.

It has been proposed that early adolescence is the period which is particularly susceptible to drug induced alterations in behavioral responses, but this age-specific hypothesis has yet to be fully investigated. To examine if this hypothesis is valid, we assessed the effects of nicotine exposure during various stages of development on nicotine-induced reward using the CPP model. We also exposed a group of adult mice to nicotine and tested them in the CPP model seven weeks later to see if the enhanced reward was an effect of previous nicotine exposure alone or if the effect was indeed unique to the adolescent phase. Results from these studies are presented in Figure 22. Once again, in both the late adolescent and adult models, no preference was seen when mice were conditioned with saline. Conditioning the mice with nicotine (0.5 mg/kg) did result in a preference for the drug-paired side, but again no differences were detected based on prior nicotine exposure.

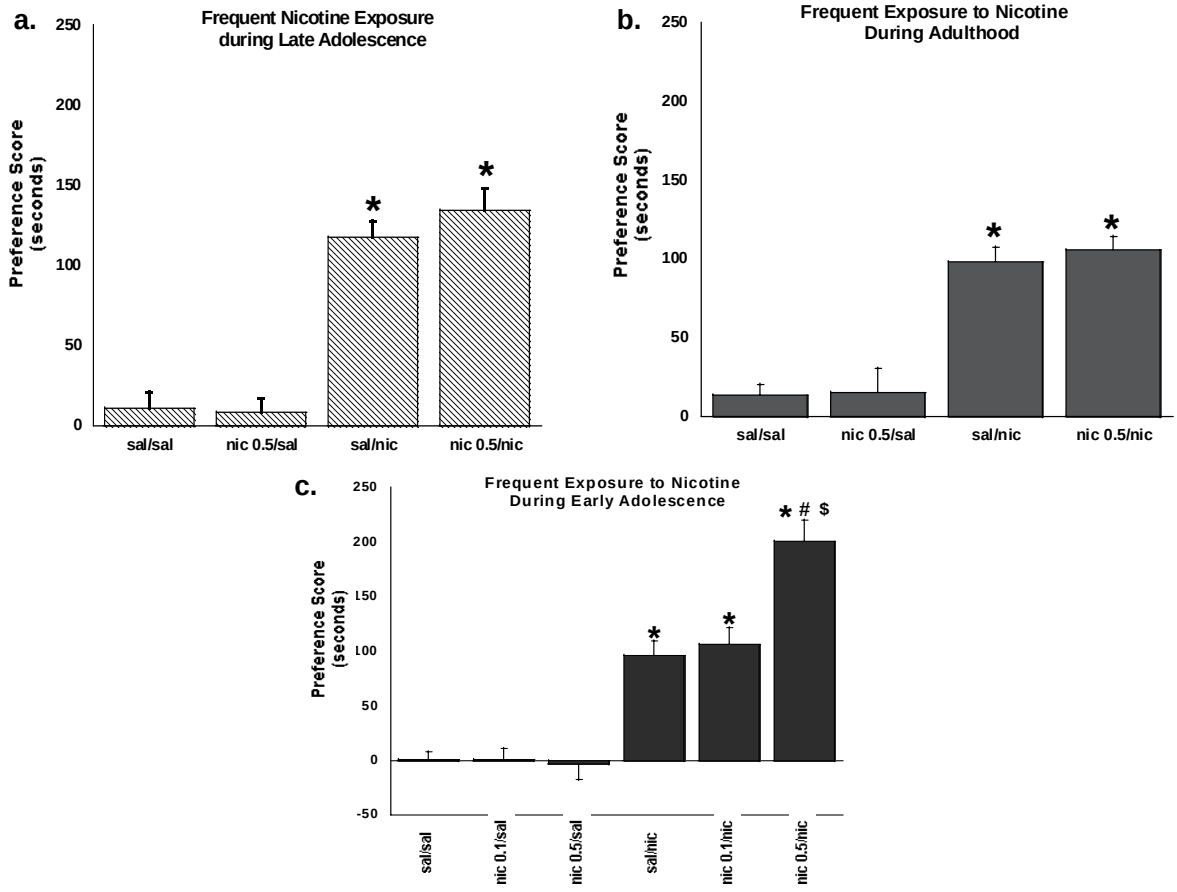

Figure 22. Effect of late adolescent and adulthood nicotine exposure on nicotine-induced reward. The y-axis represents preference score and the x-axis expresses adolescent treatment followed by treatment in the CPP paradigm. Nicotine exposure in late adolescence (a) and adulthood (b) does not elevated nicotine-induced rewarding effects later in development as measured by a CPP model. * p<.05 from respective saline control; # p<.05 from sal/nic group in the same graph

Repeated Nicotine Withdrawal Studies

The effects of adolescent nicotine exposure on nicotine withdrawal were also investigated. For these experiments, mice were evaluated for withdrawal signs twice; once during an adolescent phase and once as adults. Figure 23 shows withdrawal data from a 7 day mini-pump infusion at a dose of 48 mg/kg/day in early adolescent and adult mice. As expected from previous data shown in Chapter 2, adolescent mice (PND 21) displayed significantly attenuated somatic signs of withdrawal (Fig. 23a) as compared to adult mice (PND 70). In a hyperalgesia measure (Fig. 23c), adults demonstrated withdrawal while adolescents failed to do so. Anxiety-like behavior, an affective sign of withdrawal, was also noted in adults, but not adolescents (Fig. 23b). Furthermore, when adolescents had fully developed into adults (PND 70-2nd), they continued to display an attenuation of somatic signs. There was also no indication of withdrawal in the elevated plus maze or hot plate tests. Adult mice which were tested again after 7 weeks of maturation (PND 120-2nd) continued to display withdrawal in all four measures tested.

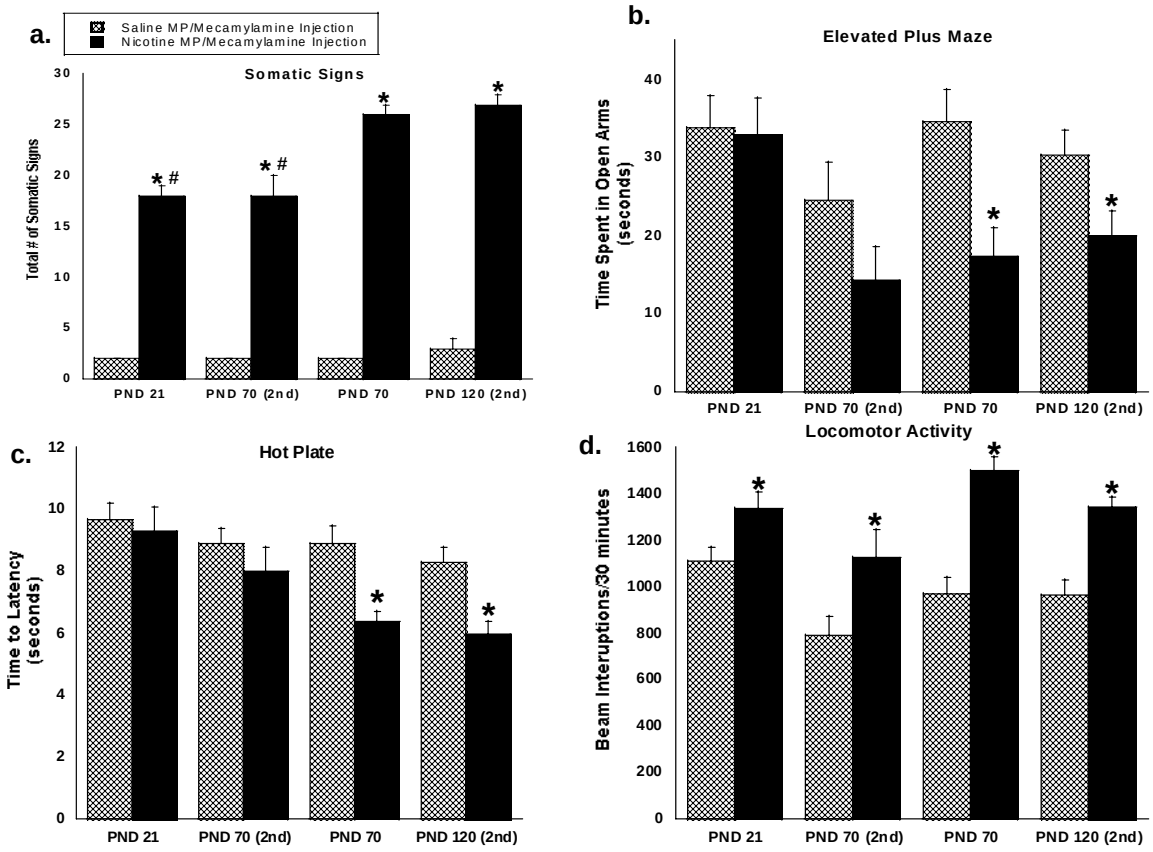

Figure 23. Effect of early adolescent nicotine exposure on nicotine withdrawal. Mice were tested for withdrawal as previously described. The x-axis denotes the age of mice upon MP implantation. PND 21=early adolescent; 1st withdrawal; PND 70 (2nd)=2nd withdrawal for early adolescent group; PND 70=adult mice; 1st withdrawal; PND120 (2nd)=2nd withdrawal for adult group. * p<0.05 from saline group and #p<0.05 from adult nicotine treatment. Each point represents the mean ± S.E. of 12 mice. MP=mini-pump, PND = postnatal day

	SS	EPM	HP	LA
PND 21- sal/sal	4 ± 1	34.8 ± 4.7	11.3 ± 1.8	952 ± 77
PND 21-nic/sal	5 ± 1	29.3 ± 5.9	9.5 ± 0.8	1141 ± 42
PND 70 (2^{nd}) sal/sal	2 ± 0	30.1 ± 1.9	10.1 ± 0.6	1053 ± 54
PND 70 (2^{nd}) nic/sal	3 ± 1	31.7 ± 1.4	9.5 ± 0.4	1101 ± 76
PND 70-sal/sal	6 ± 3	41.5 ± 0.6	9.3 ± 1.3	1102 ± 56
PND 70-nic/sal	8 ± 1	33.6 ± 6.0	9.6 ± 0.5	1154 ± 47
PND 120 (2^{nd}) sal/sal	3 ± 0	27.8 ± 2.5	8.7 ± 0.3	1055 ± 41
PND 120 (2^{nd}) nic/sal	3 ± 0	32.7 ± 2.3	8.1 ± 0.3	1006 ± 36

Table 7. Summary of control data for early adolescent repeated nicotine withdrawal study (48 mg/kg/day). Data are represented as the mean response ± S.E. of 12 mice. No significant differences were observed in any of the control groups. PND = post natal day; SS = somatic signs; EPM = elevated plus maze; HP = hot plate; LA = locomotor activity.

One of the goals of this study was to determine if all phases of adolescence represent a unique period in which nicotine exposure can lead to long-lasting behavioral effects or if this phenomenon was unique to early adolescence. Therefore, we repeated the above study using late adolescent mice which is shown in Figure 24. Unlike the early adolescent mice, the late adolescent phase did not result in the same vulnerability to lasting behavioral adaptations. Late adolescent mice displayed withdrawal signs consistent to those of adult mice in that there were no significant differences in any of the tests. Exposure to nicotine during late adolescence did not attenuate withdrawal signs once the animals had developed into adulthood.

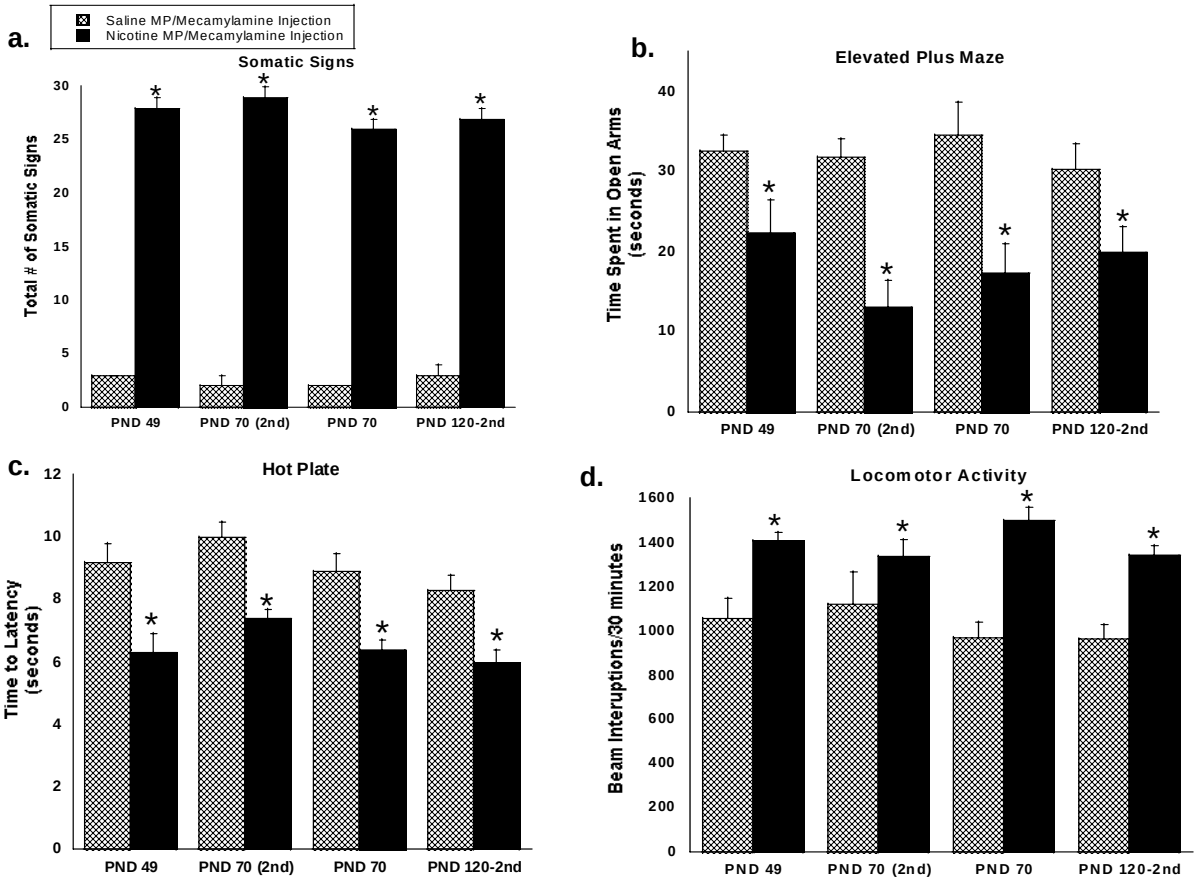

Figure 24. Effect of late adolescent nicotine exposure on nicotine withdrawal. Mice were tested for withdrawal as previously described. The x-axis denotes the age of mice upon MP implantation. PND 49=late adolescent; 1st withdrawal; PND 70-2nd=2nd withdrawal for late adolescent group; PND 70=adult mice; 1st withdrawal; PND 120-2nd=2nd withdrawal for adult group. * p<0.05 from saline group and #p<0.05 from adult nicotine treatment. Each point represents the mean ± S.E. of 12 mice. MP=mini-pump, PND = postnatal day

	SS	EPM	HP	LA
PND 49- sal/sal	4 ± 0	24.1 ± 5.4	8.5 ± 0.5	1247 ± 103
PND 49-nic/sal	3 ± 1	22.4 ± 4.4	8.2 ± 0.9	1186 ± 103
PND 70 (2^{nd}) sal/sal	3 ± 0	29.5 ± 2.1	9.2 ± 0.4	1176 ± 88
PND 70 (2^{nd}) nic/sal	3 ± 1	28.0 ± 3.0	8.4 ± 0.8	1097 ± 52
PND 70-sal/sal	4 ± 0	33.2 ± 2.7	9.1 ± 0.5	1078 ± 54
PND 70-nic/sal	3 ± 1	30.3 ± 3.2	8.6 ± 0.7	1105 ± 70
PND 90 (2^{nd}) sal/sal	3 ± 1	29.8 ± 3.1	8.4 ± 0.9	1098 ± 48
PND 90 (2^{nd}) nic/sal	2 ± 2	34.1 ± 2.9	9.0 ± 1.0	1147 ± 63

Table 8. Summary of control data for late adolescent repeated nicotine withdrawal study (48 mg/kg/day). Data are represented as the mean response ± S.E. of 12 mice. No significant differences were observed in any of the control groups. PND = post natal day; SS = somatic signs; EPM = elevated plus maze; HP = hot plate; LA = locomotor activity.

Another important component of our nicotine withdrawal studies was to examine the duration of adolescent nicotine exposure. In this study, early adolescent mice were only exposed to nicotine for 3 days prior to precipitating withdrawal. In somatic signs (Fig. 25a), withdrawal signs were noted in both age groups, but early adolescent mice continued to display a significant decrease in withdrawal intensity as compared to adults. Only adults displayed withdrawal signs in the elevated plus maze, hyperalgesia, and hyperactivity tests. Once again, when early adolescent mice were allowed to develop to adults (PND 70-2nd), they retained their attenuated level of somatic withdrawal signs.

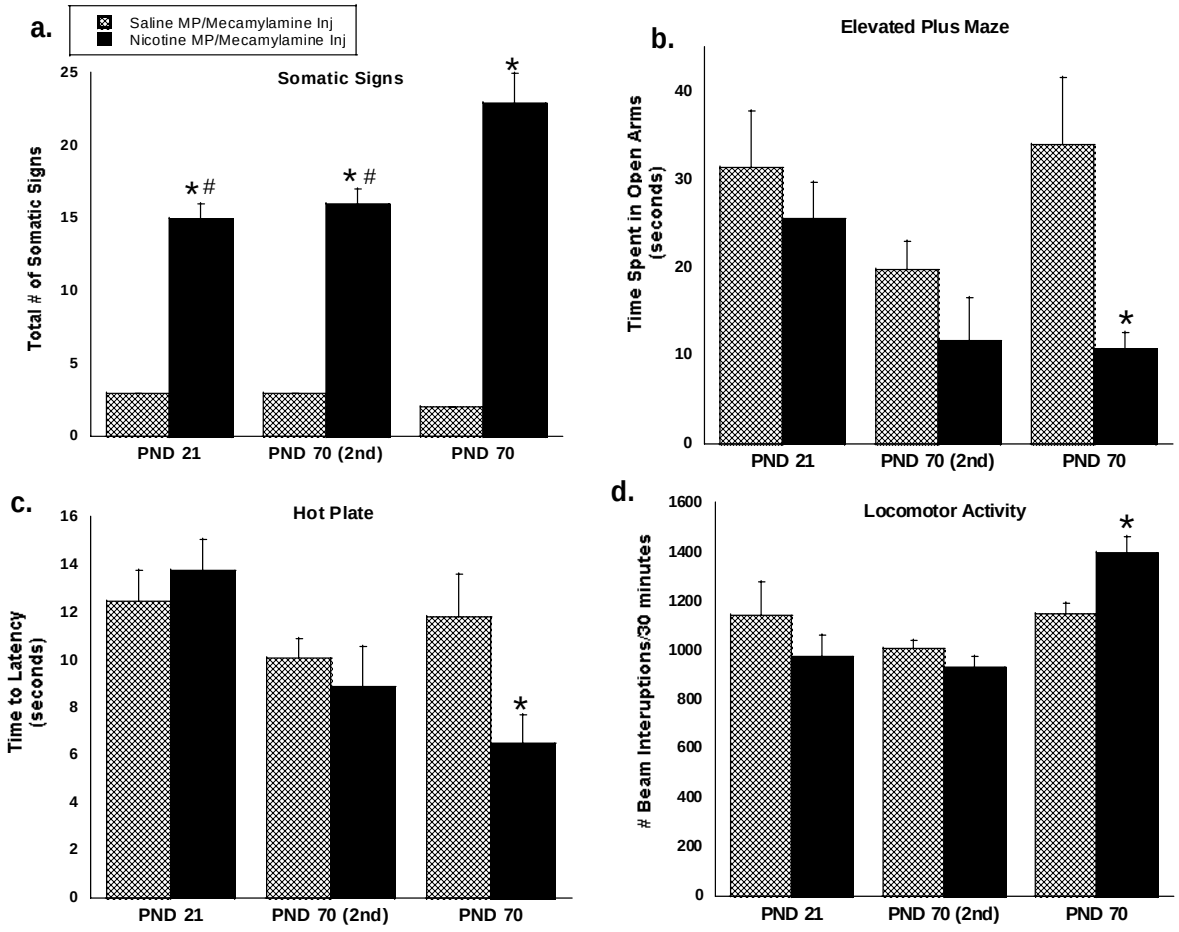

Figure 25. Three day model of early adolescent nicotine exposure on nicotine withdrawal. Mice were tested for withdrawal as previously described in the methods section. The x-axis denotes the age of mice upon MP implantation. PND 21=late adolescent; 1st withdrawal; PND 70(2nd)=2nd withdrawal for late adolescent group; PND 70=adult mice; 1st withdrawal. * p<0.05 from saline group and #p<0.05 from adult nicotine treatment. Each point represents the mean ± S.E. of 12 mice. MP=mini-pump, PND =postnatal day

Nicotine dose was also considered in these studies since it is common for adolescents to smoke less than adults. In this final study, we repeated the 7 day model of exposure but lowered the nicotine mini-pump dose to 24 mg/kg/day. Once again, we were able to consistently precipitate withdrawal in this model. Only adult mice displayed significant withdrawal signs in the plus maze and hot plate tests. Similar to our higher dose model, both age groups displayed significant somatic withdrawal signs, but adolescents showed attenuation in the withdrawal intensity (Fig. 26a). After maturing to adults, mice which were previously exposed to nicotine as adolescents continued to show a reduction in somatic signs.

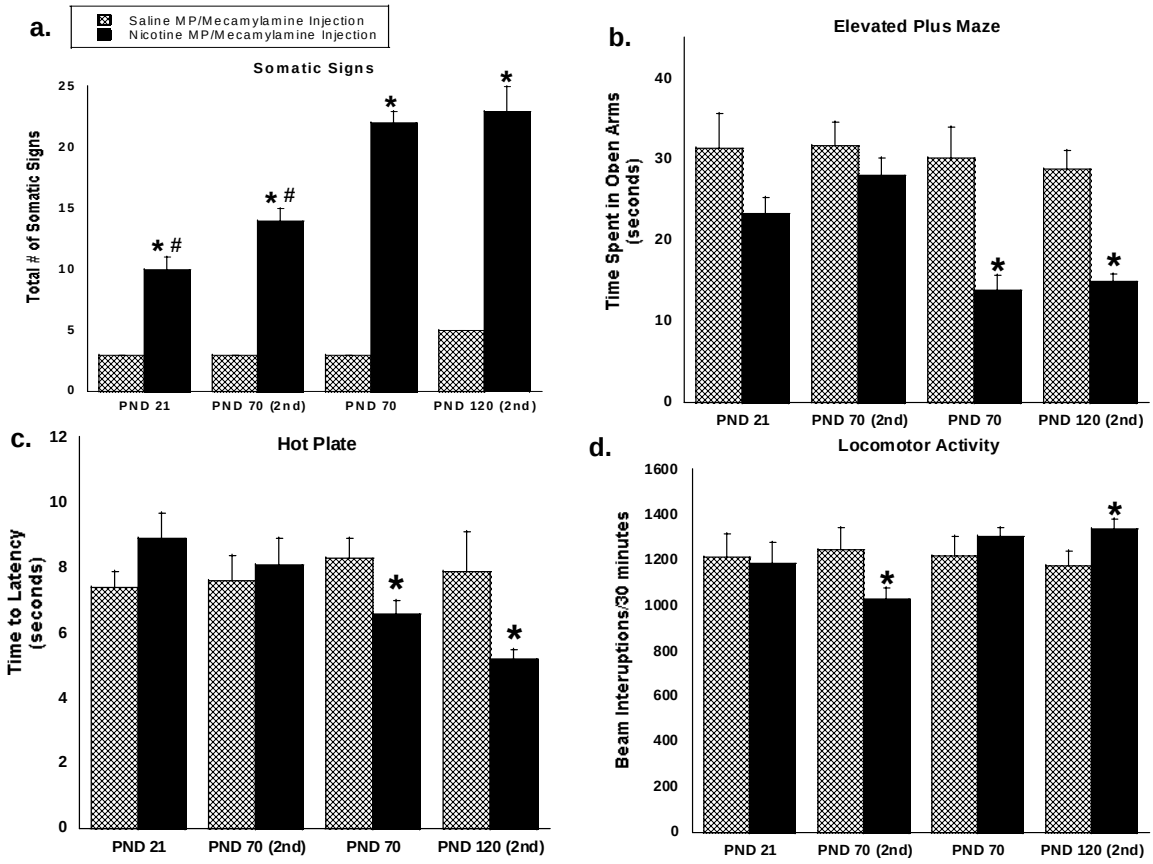

Figure 26. Effect of a low dose of early adolescent nicotine exposure on nicotine withdrawal. Mice were tested for withdrawal as previously described with MP dose reduced to 24 mg/kg/day. The x-axis denotes the age of mice upon MP implantation. PND 21=early adolescent; 1st withdrawal; PND 70 (2nd)=2nd withdrawal for early adolescent group; PND 70=adult mice; 1st withdrawal; PND120 (2nd)=2nd withdrawal for adult group. * p<0.05 from saline group and #p<0.05 from adult nicotine treatment. Each point represents the mean ± S.E. of 12 mice. MP=mini-pump, PND = postnatal day

	SS	EPM	HP	LA
PND 21- sal/sal	3 ± 0	33 ± 2.6	7.6 ± 0.5	1182 ± 48
PND 21-nic/sal	2 ± 1	32.3 ± 3.5	7.5 ± 0.4	1072 ± 25
PND 70 (2^{nd}) sal/sal	3 ± 0	29.5 ± 2.3	7.4 ± 0.4	1293 ± 36
PND 70 (2^{nd}) nic/sal	2 ± 1	34.6 ± 3.5	8.8 ± 1	1116 ± 42
PND 70-sal/sal	3 ± 0	31.6 ± 2.2	8.2 ± 0.5	1233 ± 75
PND 70-nic/sal	2 ± 0	31.5 ± 1.9	7.7 ± 0.5	1173 ± 15
PND 120 (2^{nd}) sal/sal	2 ± 0	28.9 ± 2.1	8.0 ± 0.2	1265 ± 78
PND 120 (2^{nd}) nic/sal	3 ± 1	33.1 ± 2.7	7.3 ± 0.5	1221 ± 35

Table 9. Summary of control data for repeated nicotine withdrawal (24 mg/kg/day). Data are represented as the mean response ± S.E. of 12 mice. No significant differences were observed in any of the control groups. PND = post natal day; SS = somatic signs; EPM = elevated plus maze; HP = hot plate; LA = locomotor activity.

In Chapter 5, our data indicated that naïve adolescent mice demonstrated an enhanced functional response of neuronal nAChRs as compared to adult receptors. We wanted to investigate the effects of adolescent nicotine exposure to see if this treatment would result in long-lasting effects of receptor function that correlate with behavioral changes. Figure 27 shows the results of both adolescent and adult synaptosome stimulation after pretreatment with either nicotine or saline 7 weeks prior to testing. The cortex, hippocampus, and thalamus regions showed a dose-dependent increase in nAChR function between the 1μM and 10μM concentrations of nicotine. In three out of four regions (all except cortex), the mice which were pretreated with nicotine 0.5 mg/kg in adolescence showed a significant elevation in nAChR function as compared to their saline controls. In contrast, those mice treated with nicotine in adulthood did not demonstrate any differences as compared to the saline group. At the 10μM concentration, there was a significant increase in the adolescent mice receiving nicotine, but adult mice showed no differences based on pretreatment.

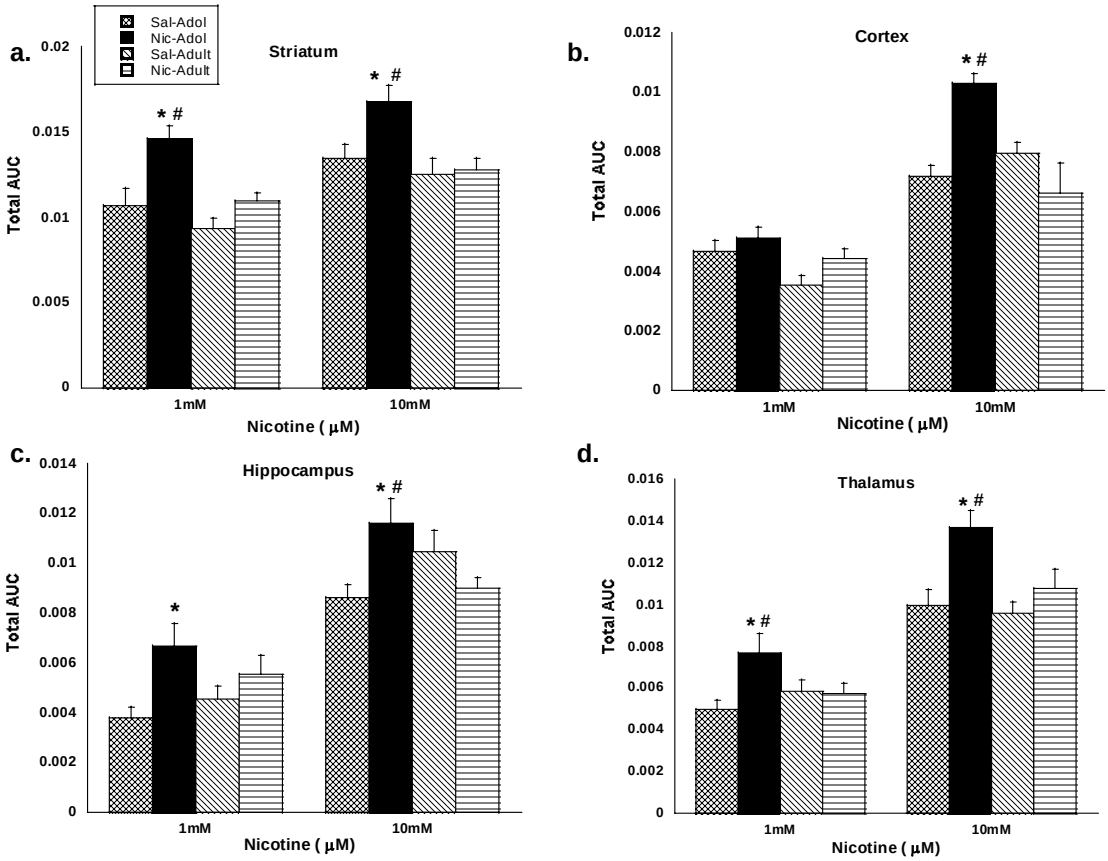

Figure 27. The effect of early adolescent nicotine exposure on nAChR function in adulthood. Two concentrations of nicotine are plotted on the x-axis and total area under the curve (AUC) is represented on the y-axis. Bars represent adolescent and adult mice which were pretreated with either saline or nicotine. Results are expressed as mean AUC ± S.E. * $p<0.05$ from respective saline control; # $p<.05$ from Adult-Nic group at same concentration.

D. Discussion

Data from these studies show that adolescent nicotine exposure affects both nicotine reward and withdrawal in adulthood. In the CPP model we have shown that early, but not late adolescent nicotine exposure elevates nicotine reward in adulthood in a dose- and duration-dependent manner. Mice which were exposed to a moderate dose of nicotine (0.5 mg/kg) in a frequent administration pattern demonstrated enhanced rewarding effects of nicotine as adults. The lower dose of nicotine (0.1 mg/kg) as well as less frequent exposure patterns did not result in the same level of enhanced reward. To eliminate the possibility that nicotine exposure can induce alterations in behavior at any age and demonstrate the selectivity of these changes, we also exposed adult mice to nicotine and tested them in the CPP model 7 weeks later. However, enhancement of rewarding effects was not seen using this paradigm. This result shows that moderate exposure to smoking during adolescence can have significant consequences in adulthood. Furthermore, it supports earlier data suggesting that early adolescence (PND 24-31) is a unique period for vulnerability to nicotine dependence.

On the other hand, nicotine exposure and withdrawal during adolescence had long-lasting effects on nicotine withdrawal in adulthood. Both short term (3 days) and long term (7 days) nicotine exposure caused persistent decreases in withdrawal signs once mice had reached adulthood. In addition, both a high (48 mg/kg/day) and low (24 mg/kg/day) dose of nicotine resulted in this same attenuation. However, we have again demonstrated that this phenomenon is unique to the early adolescent phase since late adolescent nicotine exposure did not result in a persistent decrease in withdrawal signs.

Interestingly, our results indicate that early adolescent, but not adulthood, nicotine exposure causes an elevation of nAChR function 7 weeks following injections (in adulthood). It appears that when nicotine is given in adolescence there are long-lasting effects at the receptor level which translate into changes in behavioral response. Data from this study and from the previous rubidium efflux study in Chapter 5 offer a potential mechanism by which nicotine induced persistent alterations in levels of dependence. However, mechanisms of the persistent increase in receptor function are not clear since binding studies were not conducted.

Our data imply that early adolescence is a critical period in becoming dependent on nicotine for a lifetime. Even short periods of exposure to cigarette smoking, which are often seen in the adolescent population, could have long-lasting and detrimental effects on smoking behavior. Studies from the World Health Organization show evidence that around 50% of those who start smoking in adolescent years go on to smoke for 15 to 20 years (2002). These statistics should indicate the critical nature of providing influential prevention messages at an early age. The longer a child or teenager is prevented from smoking or exposure to nicotine, the higher the chance of preventing lifetime dependence. Furthermore, the issue of secondhand smoking should be considered. Indeed, human studies show that adolescents who are exposed to secondhand smoke are more likely to develop chronic health issues such as asthma (Tager 2008) and earaches (Lee et al. 2003), but how this type of exposure affects nicotine dependence in those children has yet to be explored. Our results could have important implications in prevention messages and even policy making.

THE EFFECTS OF ADOLESCENT NICOTINE EXPOSURE ON COCAINE-INDUCED BEHAVIORAL RESPONSES

A. Introduction

In addition to adolescent nicotine exposure increasing lifetime nicotine dependence, several studies have investigated the possibility of nicotine serving as a drug which will lead adolescents to further illicit drug use later in life. Indeed, nicotine is one of the first and most commonly abused drugs in adolescence and is known to be a strong predictor of subsequent alcohol and other drug abuse (Kandel et al. 1992). Furthermore, the adolescent period is one of dramatic structural changes involving synaptic pruning, apoptosis, and cell migration (Huttenlocher 1984; Lidow and Rakic 1992). Adolescent nicotine exposure is thought to cause alterations in brain structure and function as well as changes in the mesolimbic reward pathway which is highly involved in drug addiction (Slotkin 2004). Specifically, researchers have demonstrated nicotine's ability to alter important neurotransmitter systems such as the serotonergic, glutaminergic, cholinergic, and dopaminergic among others (Trauth et al. 2000; Xu et al. 2002; Adriani et al. 2004). For example, adolescent rats (PND 30 to PND 47) given nicotine via mini-pump demonstrated a decrease in serotonergic receptors ($5HT_2$) in the hippocampus and cerebral cortex (Xu et al. 2002). Adriani et al. (2004) measured levels of AMPA GluR2/3 subunits, thought to be involved in the control of addictive behaviors two months following adolescent nicotine exposure. The results showed a dose-dependent downregulation of these subunits in the striatum and hippocampus, but comparable exposure during adulthood had either opposite or no effects.

These structural alterations often lead to changes in behavioral responses to other drugs of abuse as well. For example, adolescent nicotine exposure resulted in long-lasting changes in the rewarding properties of cocaine and alcohol (Kelley and

Middaugh 1999; Kelley and Rowan 2004). Kelley and Rowan (2004) found that adolescent mice exposed to nicotine (0.3, 1.0, or 3.0 mg/kg) from PND 25-57 showed a decreased in response to cocaine's rewarding effects when tested after a 28 day drug-free period. On the other hand, mice demonstrated an increased response to cocaine's locomotor activating effects. Additionally, McQuown et al. (2006) showed that in rats, i.v. pretreatment with nicotine (0.03 mg/kg/0.1ml) in adolescence for 4 days resulted in enhanced cocaine-reinforced responding.

Cocaine and nicotine share common neuronal mechanisms which could suggest that adolescent nicotine exposure can result in alterations to behavioral responses to cocaine. Results of three earlier studies suggest such implications (Kelley and Middaugh 1999; Kelley and Rowan 2004; McQuown et al. 2006). However, these previous studies have not investigated these effects under the same conditions. Moreover, they have not addressed important considerations such as dose, duration of exposure, and route of administration. In this set of studies, we have characterized the effects of adolescent nicotine exposure on three separate cocaine-induced behaviors in mice which represent different aspects of cocaine dependence. It is important to examine a variety of behaviors in the same species and under the same pretreatment conditions in order to gain a more complete understanding of these effects. First, we examined both high and low doses of nicotine, as well as duration of nicotine exposure, on cocaine-induced reward using the CPP model. Second, we evaluated the effects of these parameters on cocaine's acute effects using locomotor activity testing. Finally, we investigated locomotor sensitization to cocaine in pretreated animals since this model has been established as a good indicator of neuronal plasticity effects (Robinson and Becker 1986).

B. Methods

Drugs

(-)-Nicotine bitartrate and mecamylamine hydrochloride were purchased from Sigma Chemical Company (Milwaukee, WI). All doses are expressed as free base. Cocaine was provided by the National Institute for Drug Abuse.

Adolescent Injection Protocol

Mice received nicotine during early adolescence (PND 21-31), late adolescence (PND 49-59) or adulthood (PND 70+). Based on studies in Chapter 6, we choose to only use either an acute pattern (1 day) or a repeated pattern of exposure (7 days) in duration. Nicotine (0.1 and 0.5 mg/kg) or saline was administered s.c. twice daily with injections being approximately 6 hours apart (9am and 3pm). Mice were kept in their home cages and allowed to mature until they had reached adulthood at which point they were evaluated in paradigms as described below.

Conditioned Place Preference Studies

Once adolescent mice had reached adulthood (PND 70), they were tested for cocaine reward using conditioned place preference. The precise protocol for conditioned place preference was the same as previously described in Chapter 2. Briefly, mice have a pre-conditioning day which is a drug free assessment of baseline preference in a three compartment chamber. This is followed by three days of conditioning to either nicotine or cocaine. Only one conditioning dose of cocaine was used in this model (10 mg/kg i.p.). The final day of the paradigm is the same as day 1 and assesses preference after the conditioning period. Preference scores are expressed

as time spent on drug-paired side minus time spent on saline-paired side. A positive number indicated a preference for the drug-paired side, while a negative number indicated an aversion to the drug-paired side. A number at or near zero indicated no preference for either side.

Acute Locomotor Activity

Another group of mice were tested for cocaine-induced hyperactivity using locomotor chambers after reaching adulthood. Dose-response curves were generated for each pretreated group (saline, nicotine 0.1 mg/kg, or nicotine 0.5 mg/kg). Mice were injected i.p with saline or various doses of cocaine (5, 10, and 15 mg/kg) and then placed into individual Omnitech photocell activity cages (Columbus, OH; 28 x 16.5 cm) 10 min after injection. Mice were allowed to habituate to the chamber for 5 minutes before data collection began. Interruptions of the photocell beams (two banks of eight cells each) were then recorded for the next 30 min in 10 min intervals. Data are expressed as number of photocell interruptions.

Cocaine Locomotor Sensitization

For this study, only early adolescent mice (PND 22-28) were pretreated with saline or nicotine (0.5 mg/kg) injections. Our protocol was based on the study by Biala (2003). Briefly, once the mice had reached PND 70, a 13 day cocaine sensitization protocol began. On day 1, mice were given a saline injection (i.p.) and placed into locomotor activity chambers for a 30 minute habituation period. Immediately following, mice were removed from the chambers and activity counts were recorded. Mice were randomly divided into three groups: saline-saline, saline-cocaine, and

cocaine-cocaine (groups represent the acquisition day drug followed by the challenge day drug). Mice were then given another injection of either saline or cocaine 20 mg/kg (i.p.), depending on the assigned group, and placed in the chambers again for a 30 minute acquisition period. This procedure was repeated on days 2-5. Days 6-12 were considered a drug free week in which the animals were not given injections or exposed to the chambers. On day 13, mice were tested again in the same manner as described for days 1-5, but cocaine mice received a challenge dose of cocaine of 5 mg/kg (i.p.). Counts were recorded after a 30 minute test period.

C. Results

Effect of Adolescent Nicotine Exposure on Cocaine-Induced Conditioned Place Preference

Figures 28-30 show the results of our cocaine-induced CPP study after mice had received nicotine at various stages of development. In Figure 28, mice received either an acute (1 day) or repeated (7 day) exposure to nicotine during early adolescence. All mice which were conditioned with cocaine in the CPP model developed significant preference for the drug-paired side as compared to their respective saline controls. Interestingly, mice which had a 7 day exposure to the higher dose of nicotine (0.5 mg/kg), displayed a significantly enhanced level of preference as compared to those mice which were pretreated with saline. In addition, this group showed significantly enhanced preference as compared to the same treatment group in the late adolescent study (Figure 29) as indicated by the $ symbol in Figure 28. On the other hand, the lower dose of nicotine (0.1 mg/kg) did not produce a significant enhancement of

reward. No differences were noted in the acute exposure paradigm. The results of cocaine-induced CPP following late adolescent and adult nicotine exposure are shown in Figures 29 and 30 respectively. As expected, all mice conditioned with cocaine during CPP testing displayed significant preference for the drug paired side. In contrast to data in early adolescent mice, late adolescent mice did not demonstrate any significant differences based on pretreatment status in either the acute or repeated exposure protocol. Similarly, mice which received nicotine exposure during adulthood displayed approximately equal levels of preference for cocaine despite varying pretreatment groups. These results indicate that the enhancement of cocaine-induced preference is unique to the early adolescent period and is not due to previous nicotine exposure alone.

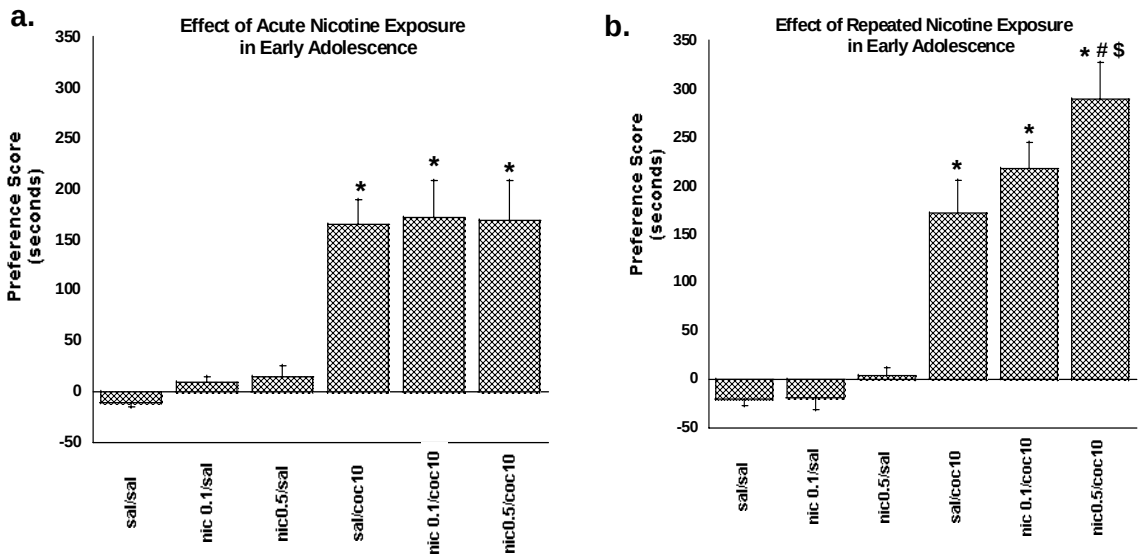

Figure 28. Effects of early adolescent nicotine exposure on cocaine-induced CPP in adulthood. The y-axis represents preference score and the x-axis expresses adolescent treatment followed by treatment in the CPP paradigm. Repeated nicotine exposure in early adolescence elevated cocaine-induced rewarding effects in adulthood. * p<.05 from respective saline control; # p<.05 from sal/nic group in the same graph; $ see text in results section

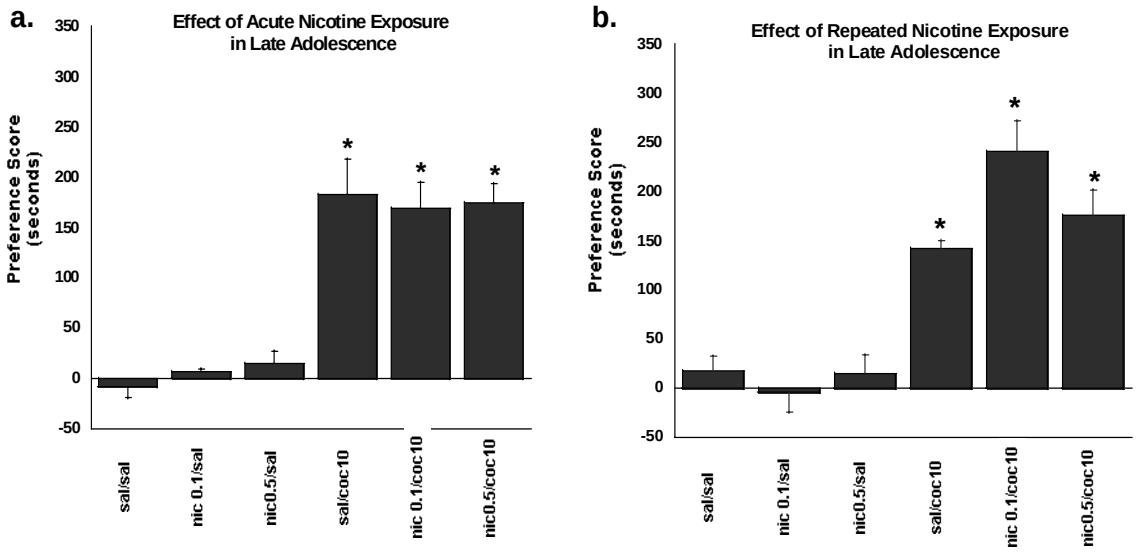

Figure 29. Effects of late adolescent nicotine exposure on cocaine-induced CPP in adulthood. The y-axis represents preference score and the x-axis expresses adolescent treatment followed by treatment in the CPP paradigm. * p<.05 from respective saline control

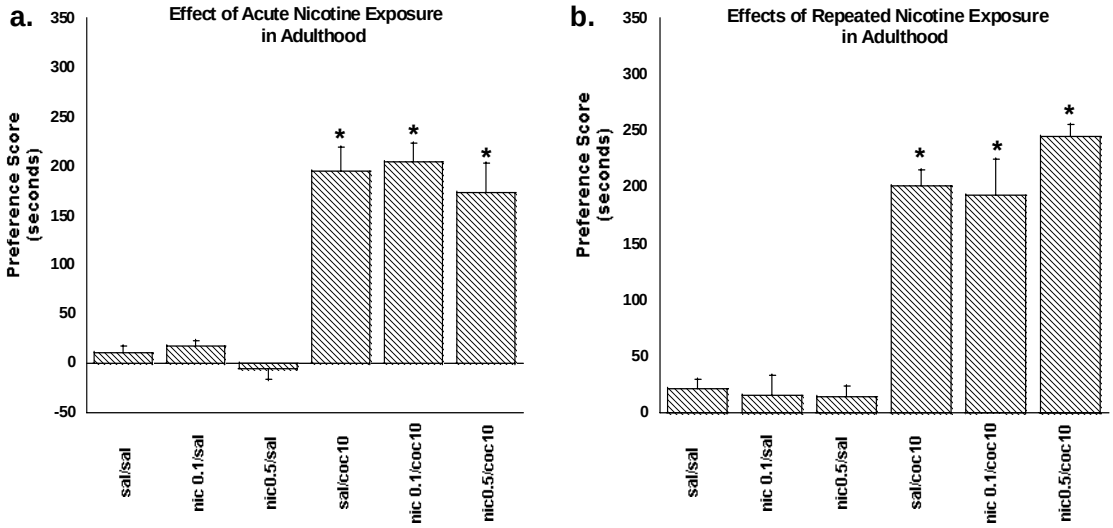

Figure 30. Effects of adulthood nicotine exposure on cocaine-induced CPP 7 weeks later. The y-axis represents preference score and the x-axis expresses adolescent treatment followed by treatment in the CPP paradigm. * p<.05 from respective saline control

Effects of Adolescent Nicotine Exposure on Cocaine-Induced Hyperactivity

In this study, we examined the effects of adolescent nicotine pretreatment on cocaine's acute effects using a locomotor activity test. Figures 31-33 show the results from these studies. Figure 31 depicts the results from both an acute (1 day) and repeated (7 day) nicotine exposure pattern during early adolescence. No changes were observed after acute exposure; however those mice which were pretreated with the higher dose of nicotine (0.5 mg/kg) in early adolescence displayed a significant elevation in cocaine-induced hyperactivity as compared to those pretreated with saline or a low dose of nicotine (0.1 mg/kg). Figures 32 and 33 show the results from studies where pretreatment occurred in late adolescence and adulthood respectively. No significant differences were seen based on pretreatment injections in either age group confirming that the effect seen in Figure 31 is unique to the early adolescent period. Interestingly, saline treated mice undergoing repeated nicotine exposure displayed a trend for slightly decreased activity. This was consistent across all three age groups and is likely due to the stress of repeated injections since this behavior was not seen after acute exposure.

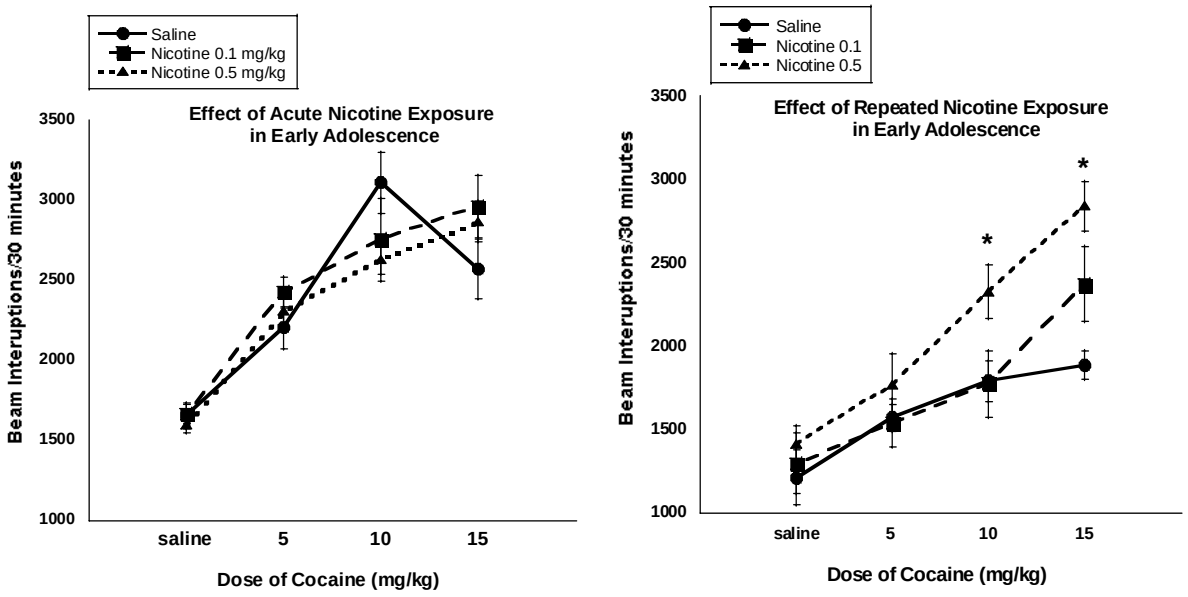

Figure 31. Cocaine-induced hyperactivity following nicotine exposure in early adolescence. Mice were pretreated with saline or nicotine during early adolescence either acutely (1 day) or repeatedly (7 days) and were tested for cocaine hyperactivity in adulthood. n=6/group *p<0.05 from saline pretreatment.

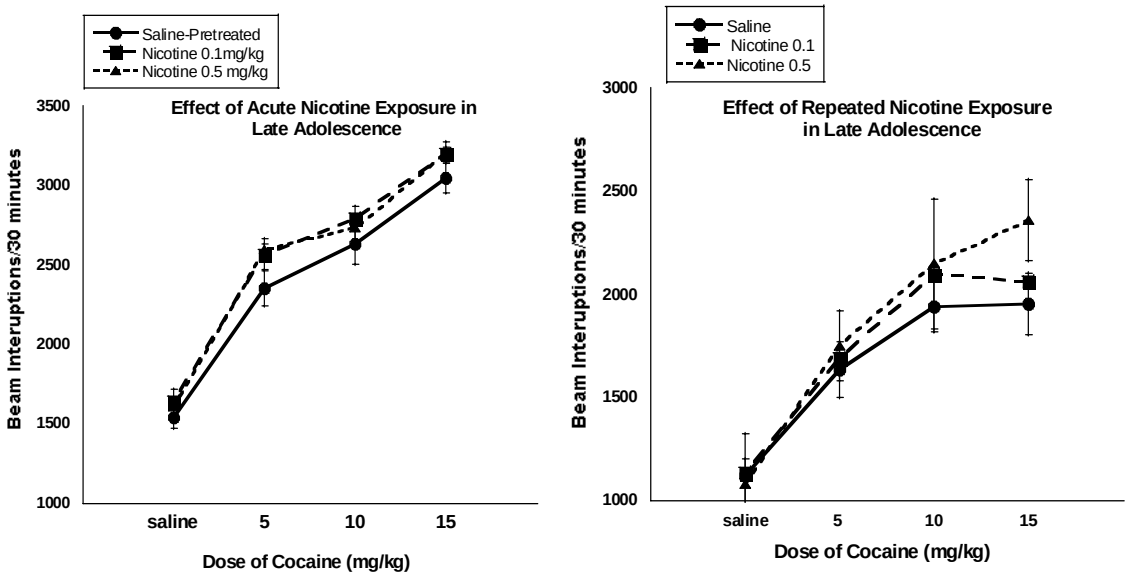

Figure 32. Cocaine-induced hyperactivity following nicotine exposure in late adolescence. Mice were pretreated with saline or nicotine during late adolescence either acutely (1 day) or repeatedly (7 days) and were tested for cocaine hyperactivity in adulthood. n=6/group

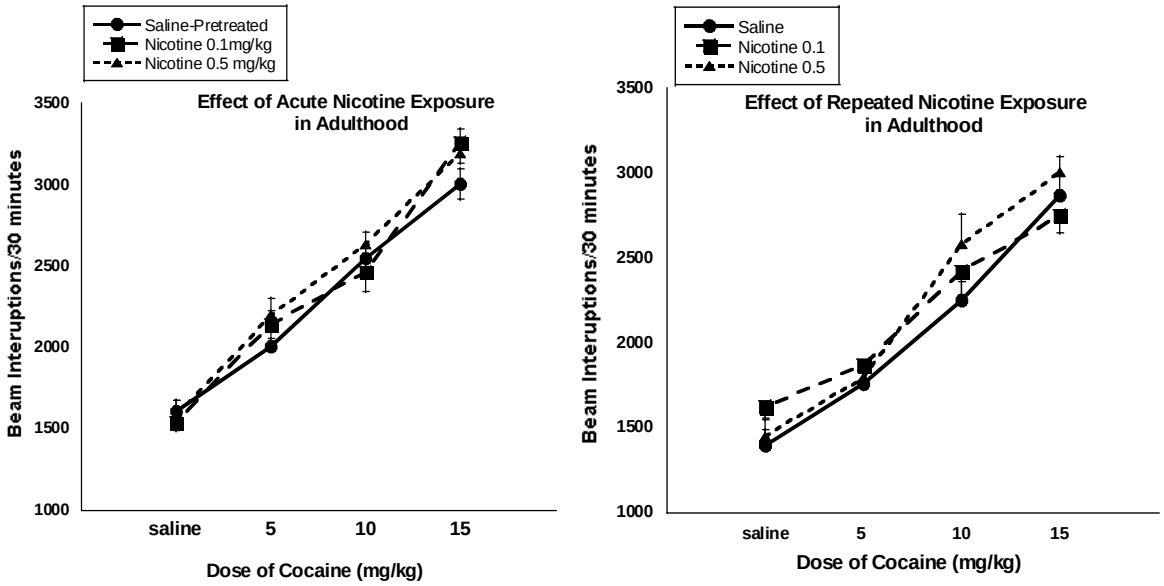

Figure 33. Cocaine-induced hyperactivity following nicotine exposure in adulthood. Mice were pretreated with saline or nicotine adulthood either acutely (1 day) or repeatedly (7 days) and were tested for cocaine hyperactivity 7 weeks later. n=6/group

Effects of Adolescent Nicotine Exposure on Locomotor Sensitization to Cocaine

Finally, results from Figure 34 show our study examining the effects of early adolescent nicotine treatment on cocaine-induced behavioral sensitization. Mice which were pretreated with nicotine in adolescence are shown in the solid bars while saline pretreated mice are shown in the non-solid bars. During the acquisition period, mice which were treated with cocaine (20 mg/kg) demonstrated enhanced locomotor activity as expected with no differences due to adolescent pretreatment (*$p<.05$ as compared to sal-sal). On challenge day two groups received an injection of cocaine i.p. (5 mg/kg). Both saline and nicotine pretreated mice who were treated with cocaine during acquisition displayed enhanced locomotor activity as compared to those mice treated with saline during acquisition. However, mice which were pretreated with nicotine in adolescence showed a significant elevation in cocaine-induced locomotor activity as compared to saline pretreated animals. These results show that we were able to induce locomotor sensitization to cocaine and that early adolescent nicotine exposure enhances this effect.

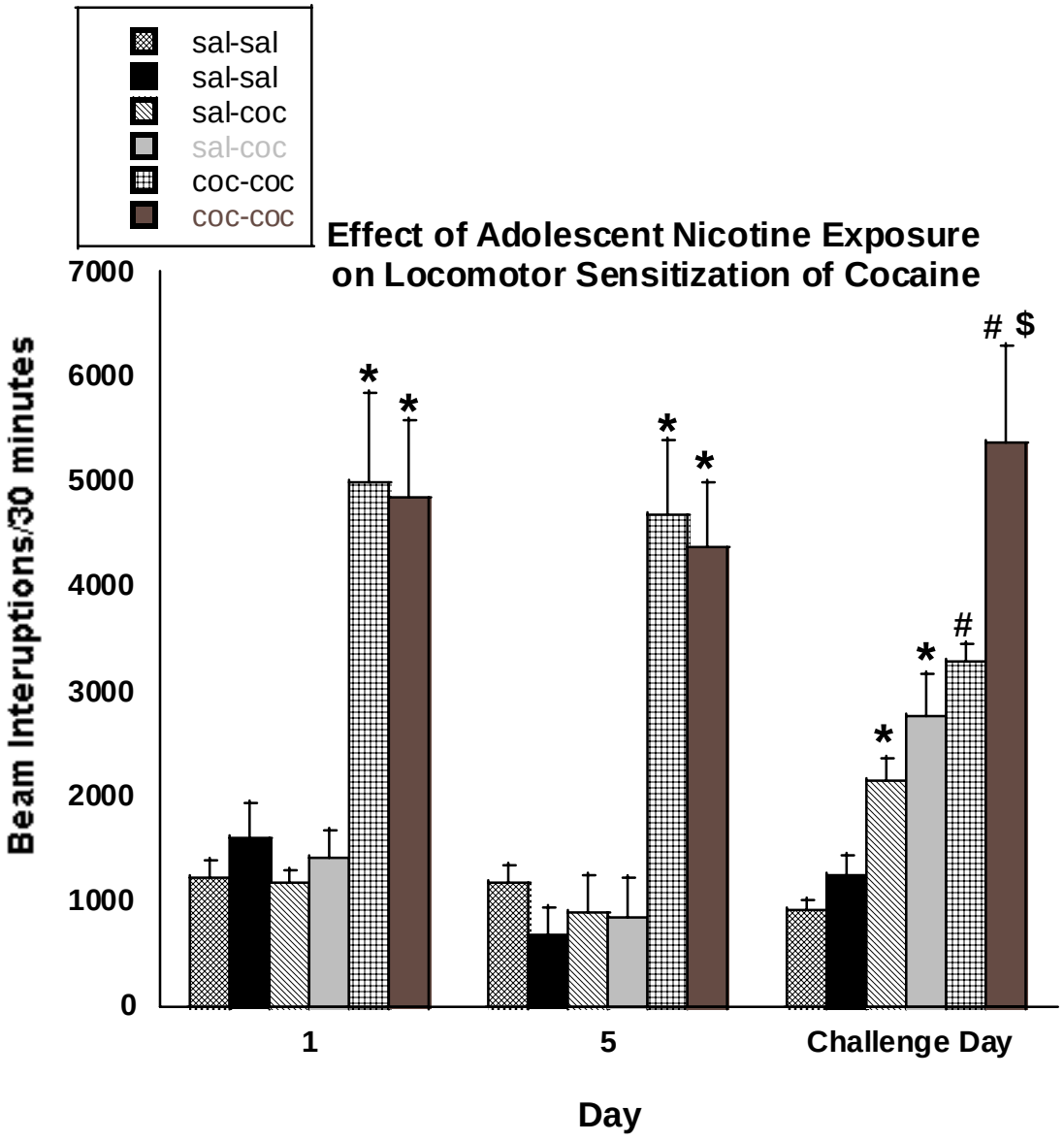

Figure 34. Cocaine-sensitization in ICR male mice. Early adolescent mice were pretreated with either saline (non-solid bars) or nicotine (solid bars) for 7 days and were tested for cocaine-induced locomotor sensitization in adulthood. Treatment groups are represented by acquisition drug-challenge drug in the legend (ex. sal-coc = saline during acquisition and cocaine on challenge day) *p<.05 from sal-sal control on the same day; # p<.05 from sal-coc group; $p<.05 from saline pretreated coc-coc group.

D. Discussion

The use of tobacco often begins during the adolescent period. Furthermore, it is well-established that the commencement of smoking at a young age correlates with a higher prevalence of nicotine dependence in adulthood (Colby et al. 2000; Kandel and Chen 2000). However, there is not as much evidence for the effects of cigarette smoking on dependence for other drugs of abuse. Some studies have shown that nicotine is a strong predictor of subsequent drug use (Kandel et al. 1992), but the exact mechanism behind these changes is unknown.

Specifically, we have chosen to investigate the effects of adolescent nicotine on cocaine-induced behavior. To date, studies have shown mixed results in this regard, but several factors, such as different models, strains, and species, could contribute to these differences. We decided to utilize several paradigms in order to investigate the consistency of nicotine's effects in mice. In a CPP model of reward, our results demonstrated that the higher dose of nicotine (0.5 mg/kg) given for 7 days enhanced preference for a cocaine-paired environment. In contrast to our findings, Kelley and Rowan (2004) found that C57BL/6J mice demonstrated a decrease in cocaine-induced reward as measured by CPP after 25 days of adolescent nicotine exposure. This discrepancy could be accounted for by the difference in mouse strain (C57BL/6J vs. ICR) as well as length of exposure (25 days vs. 7 days). Interestingly, they noted that this exposure led to an increase in cocaine's motor activating effects which is in agreement with the results from our acute locomotor study. Other studies in rats which have utilized a shorter duration of adolescent nicotine exposure have found that the

rewarding effects of cocaine are enhanced. Indeed, McQuown et al. (2006) reported that a low dose of nicotine treatment for four days in adolescence enhanced the reinforcing effects of cocaine in an i.v. self-administration model using a FR1 schedule. Similarly, rats given nicotine from PND 35 to 44 demonstrated an enhancement of cocaine-induced reward using a CPP paradigm (McMillen et al. 2005).

Our findings in the CPP model strongly suggest that nicotine during adolescence may act to cross-sensitize the brain to cocaine's rewarding effects. Indeed adolescence is a unique period of brain maturation and development. Much of the motivational circuitry controlling reward and reinforcement is still undergoing alterations (Chambers et al. 2003). Specifically, dopaminergic projections from the PFC to the NAc may be influenced by this exposure which would have effects on the pleasurable experiences associated with drugs of abuse such as cocaine. In addition, nicotine may be acting directly on the maturing dopamine system (Andersen 2003). It has been established that nicotinic receptors play in a role in regulation of dopaminergic neuronal projections (Cragg 2006) and it is possible that nicotine exposure during a critical period such as adolescence may yield long-lasting changes in this system. Indeed, quantity of dopamine transporters will be important to examine since this serves as a major target for cocaine.

Also in agreement with our reward studies is data from our locomotor sensitization model which is linked to the establishment of drug dependence. We have found that a 7 day nicotine pretreatment in early adolescence enhanced sensitization to cocaine on challenge day as compared to saline pretreatment. To our knowledge, this is

the first study to examine the effect of adolescent nicotine exposure on cocaine-induced sensitization in mice. These results suggest that early nicotine exposure may correlate with an increased risk of relapse after a period of withdrawal and further implicate a role for dopamine in the cross-sensitization to other drugs of abuse. Implications to human studies are also evident. For example, adolescents who had previously smoked cigarettes were found to have higher initial "wanting" scores and were more likely to become cocaine-dependence than non-smokers (Lambert et al. 2006).

It is of interest to note that an acute exposure to nicotine did not elicit the same effects as a repeated exposure pattern in our CPP and acute locomotor models. This finding implies that long-lasting alterations in neurochemical systems require activation of targets involved in synaptic plasticity or gene expression. The importance of this observation will be further addressed in the general discussion. Taken together, results from this chapter support the hypothesis that adolescent nicotine exposure is able to enhance susceptibility to other drugs of abuse as well. Once again, this result stresses the importance of preventing adolescent experimentation with tobacco as it can rapidly cause persistent changes in drug-induced behavioral responses.

GENERAL DISCUSSION

A. Rationale and Summary of Overall Hypothesis

In the United States, smoking-related illnesses cause more than 430,000 deaths and cost more then $150 billion annually (CDC MWRR 2002). Most of these lifetime smokers begin smoking during adolescence (Chassin et al. 1990). It is common for teenagers to explore tobacco and alcohol use during the developmental phase of adolescence; however many adolescent smokers show loss of autonomy over nicotine after just a few cigarettes (DiFranza 2002) despite the desire to quit (Eissenburg and Balster 2000). Factors such as cravings and withdrawal symptoms are frequently cited by this age group as reasons for unsuccessful quit attempts (Johnson 1982; Biglan and Lichtenstein 1984). Indeed, Colby et al. (2000) wrote a review suggesting that the current methods and approaches to smoking cessation in adolescence need further attention since successful cessation rates are modest. It is imperative that better smoking cessation therapies and prevention messages are targeted specifically toward adolescents in order to decrease the number of smokers in the United States.

While the issue of adolescent nicotine dependence has recently become a focus of addiction research, there is still much work that needs to be done. To date, we do not fully understand the mechanisms which underlie an adolescent's heightened vulnerability to nicotine dependence. Furthermore, the scope and extent of the changes in the neurobiology of adolescent smokers has yet to be determined. Learning more about these changes will help us to target key areas which are affected by nicotine and to develop therapies which address these issues. To this aim, our studies have focused

on the mechanisms of change which are involved in alterations of nicotine dependence. Specifically, we decided to center our studies on the initial target of nicotine, the nicotinic acetylcholine receptor in the brain.

The work in this dissertation addresses three areas of research and contributes to the further understanding of adolescent nicotine dependence. First, we performed a full characterization of nicotine dependence in various age groups and both sexes to address age- and sex-related disparities. Second, we examined possible molecular mechanisms which may underlie these differences. Third, we sought to investigate the effects of adolescent smoking on future, and perhaps long-term, drug abuse. Overall, we hypothesized that vulnerability to nicotine dependence in adolescence is due to a shift in the balance between two key components of nicotine dependence, namely reward and withdrawal, and that this shift is due to nicotine-induced, region-specific changes in the brain. Furthermore, we predicted that nicotine exposure in adolescence would lead to long lasting changes in nicotine-induced behavior as well as dependence on other drugs of abuse.

B. Nicotine reward and withdrawal are age- and sex- dependent

Studies from our first specific aim demonstrate that two key aspects of nicotine dependence, reward and withdrawal, are both age- and sex-dependent. To our knowledge, this is the first study to characterize both of these variables under the same experimental conditions and in parallel. The data from our studies show that early adolescence is a particularly vulnerable period for developing nicotine dependence, yet this susceptibility differs for each sex. In adolescent males, the rewarding effects of

nicotine are enhanced as compared to adults while withdrawal signs are attenuated (Figures 2, 4, 6, and 8). In contrast, we found that females demonstrated enhanced nicotine withdrawal effects, but an overall decrease in nicotine reward sensitivity (Figures 2, 5, 7, and 9). There are important implications for these findings in the realm of nicotine dependence. In adolescent females, it appears that withdrawal effects are the greater contributor to long-term smoking behavior because they prohibit effective smoking cessation attempts. In fact, in agreement with our rodent studies, clinical findings report that women are less likely to quit smoking successfully due to high withdrawal effects (Leventhal et al. 2007). On the other hand, male data revealed that adolescents are more likely to continue cigarette smoking due to the reinforcing effects of the drug as shown by an increased sensitivity to low doses of nicotine in the CPP model. These studies show that smoking cessation therapies need to target the molecular mechanisms which are responsible for the most reinforcing stimuli. In males, key pathways involved in nicotine reward need to be the focus of therapeutic strategies, while in females, targeting the nicotinic receptors which are highly involved in withdrawal effects may be more critical.

In addition to sex-dependent differences, our work clearly revealed age-dependent differences in the intensities of important aspects of nicotine dependence. For example, we have shown that adolescents, given the same level of nicotine as adults, do express withdrawal signs; an important observation from a clinical perspective. Although the intensity of nicotine withdrawal in adolescents is less than that of the adult in males, this finding still confirms that adolescent smokers show signs

of nicotine dependence. It is also interesting to note that in practice, adolescent smoking intake behavior is not consistent with that of adults and it is likely that their actual nicotine intake is lower. In this regard it is difficult to make a valid assessment of how withdrawal symptoms contribute to dependence. In addition to withdrawal signs, we have shown that during adolescence positive rewarding effects of nicotine are enhanced in the male sex. It could be argued that these positive effects contribute more to the enhanced vulnerability to nicotine addiction in males since there is a high desire for immediate positive reinforcement without proper assessment of risk during the adolescent period.

C. Early adolescence presents a unique period of vulnerability to drug dependence

Several studies have demonstrated that adolescence as a whole is an important period in the development of drug dependence. However, not many studies have examined the specific phases of adolescence and how each plays a role in this enhanced vulnerability. Our work is the first to investigate the importance of adolescent phase in both reward and withdrawal models. These studies were only conducted in males as this sex was chosen to be the focus of our project. Our findings confirm that in male mice, the early adolescent phase is a unique time of development which is particularly vulnerable to the effects of nicotine. In a CPP model, only early adolescent mice demonstrate an increased sensitivity to low doses of nicotine (Figure 14). In addition, data from Figure 15 reveal that late adolescent mice exhibit the same intensity of withdrawal as adults while early adolescent have decreased withdrawal intensity in both somatic and affective signs. In agreement with our work, several studies in rats have

shown that only early adolescent rodents develop preference to nicotine (Adriani et al. 2002; Belluzzi et al. 2004). Additionally, O'Dell et al. (2006) previously reported that somatic signs of nicotine withdrawal are attenuated in early adolescent male rats. Our research confirms that finding in mice and adds to it by confirming this decrease in two additional somatic signs and one affective sign.

These data contribute to the understanding of previous human research which indicates that the initiation of smoking at an early age is known to lead to increased addiction and decreased cessation rates (Colby et al. 2000; Kandel and Chen 2000). Indeed, since the brain has not reached its full maturation, it has a heightened vulnerability to aspects of nicotine dependence which will lead to life-long smoking behavior. In summary, these findings convey the importance of delaying teenage smoking as long as possible, if not preventing it completely, through better prevention messages. Additionally, clinical trials must include these younger age groups in their studies. It will also be beneficial to address the interactions of pubertal status and hormone development with such clinical treatment strategies as the effectiveness of treatment may change with these factors.

D. Pharmacological and Molecular Mechanisms Involved in Nicotine Dependence

Chapters 3 and 5 of this dissertation address specific *in vivo* and *in vitro* mechanisms which may play a role in the enhanced propensity for adolescents to become dependent on nicotine. In Chapter 3, we assessed both the acute sensitivity to nicotine as well as tolerance to nicotine in adult and adolescent mice of both sexes. These models were chosen because of the insight they provide into the differences

observed in previous behavioral models. Acute sensitivity models allow a distinction in immediate response to a drug which has implications for disparities in nicotinic receptor function and activation. Also, tolerance, or the capacity of the body to become less responsive to a drug following chronic use, is a phenomenon which has been shown to contribute to nicotine dependence (Damaj and Martin 1993; Robinson et al. 1996).

Our data show that following acute treatment with nicotine, adolescent male mice displayed a nicotine-induced antinociception compared to adults in the tail-flick test. The implication of this finding suggests that predisposition to maintain use of nicotine in adolescent males may be due to the lessening of aversive effects due to decreased sensitivity to the drug. However, since no general decrease in nicotine's acute effects was found in our studies this implies that acute sensitivity to nicotine is not a major factor. Similarly, we observed a higher degree of tolerance to nicotine-induced antinociception in adolescent male mice in the hot-plate assay suggesting that this age group would need to increase their nicotine intake in order to attain the same level of effect as an adult. This would lead to an increased smoking behavior and higher nicotine intake in adolescence resulting in increased vulnerability to nicotine dependence. Once again, the other two measures of tolerance did not show significant results in the same manner implying that tolerance is only playing a minor role in adolescent vulnerability.

In females, we observed an opposing trend in both acute sensitivity and tolerance as compared to males. Increased sensitivity to nicotine was noted in one analgesic assay and the hypothermia test in adolescents. Moreover, a lower degree of

tolerance was detected in adolescents in the hypothermia test, but no changes were seen in antinociceptive measures. Since not all tests for acute sensitivity and tolerance to nicotine reflected similar shifts, we can assume that these mechanisms are not likely to be substantial contributors to age-dependent differences in nicotine reward and withdrawal.

In addition to behavioral mechanisms, it is probable that molecular mechanisms are involved in age-related differences in nicotine dependence. Indeed, alterations in receptor number and function as well as differences in downstream signaling would affect pharmacological responses to nicotine. The goal of the experiments in Chapter 5 was to investigate these possibilities in the male sex. Some studies have shown that adolescent nicotine exposure has important molecular consequences. Schochet et al. (2005) have shown that while nicotine exposure in general upregulates genes such as c-fos, only adolescent nicotine exposure causes changes in levels of arc mRNA in the PFC. Data from Azam et al. (2007) has shown that α5, α6, and α7 mRNA levels peak during adolescence before decreasing to a steady level in adulthood. This same study also demonstrated that there are regional differences in expression of α5, α6, and β3 mRNAs with elevated levels in the substantia nigra as compared to the ventral tegmental area. It is clear that nicotine can have significant effects on brain development and maturation particularly during the adolescent period. Yet a full understanding of receptor regulation and function is still lacking. Our research aimed to contribute to the knowledge of the effects and to link molecular mechanisms to our behavioral observations.

Our results show that in four brain regions (striatum, cortex, hippocampus, and thalamus) nAChR function was enhanced in the adolescent as compared to the adult using a rubidium efflux assay. Since these results could be due to several factors including the hypothesis that there is a greater quantity of basal nAChRs in adolescent mice, we sought to answer this question by using receptor binding studies. Using various pharmacological tools, our data reveal no significant differences in total nAChR binding or binding for any specific nAChR subtype. Since the previous study by Azam et al. (2007) found differences in mRNA levels for several nAChR subtypes, this was a surprising result. However, disparities in mRNA levels do not always translate into changes in receptor expression therefore results must be interpreted with caution. In addition to receptor binding, we also investigated nicotine-induced dopamine release from striatal synaptosomes. Again, in contrast to previous findings (Azam et al. 2007), we did not see any significant differences in dopamine release. Differences between our study and the study by Azam et al., which may account for disparities in the outcomes, include assay technique (microdialysis vs. synaptosomes in our study), age of subjects (PND 7 and 14 vs. PND 28-30 in our study), and species utilized (rats vs. mice in our study).

Importantly, we have shown that adolescent nAChRs exhibit increased function when stimulated with nicotine. The precise reasons for this increase are yet to be determined, but this functional response is likely to play a role in behavioral response to nicotine. Indeed, this data correlates well with an enhanced responsiveness in the CPP model. However, it does not correlate with the data from our acute studies in which

adolescent and adult male mice displayed no significant differences in three out of four measures in responses to acute nicotine. It may be that differences in the biochemical measure are not enough to reach behavioral thresholds in a model using short exposure to nicotine such as that in our acute studies. However, a sub-chronic or chronic dosing protocol, such as that used in CPP, is enough to surpass this threshold which is why differences are seen using this model.

This increase in nAChR function is not attributable; however, to an increase in the number of basal nAChRs during the adolescent period, despite previous data indicating an increase in mRNA expression for certain receptor subtypes (Azam et al. 2007). We have also shown that dopamine release from the striatum does not differ between adult and adolescent mice; a finding in contrast to results in the previously mentioned study done in rats. It is likely that an increase in nAChR function causes alterations in downstream effectors which may play an important role in the behavioral differences. Specific possibilities will be further discussed in the future directions section of the discussion.

E. Exposure to nicotine during early adolescence has persistent effects on nicotine dependence in adulthood

The third specific aim of this dissertation was to examine the effects of adolescent nicotine exposure on long-lasting nicotine dependence. Clinical studies suggest that the earlier a person begins smoking, the more likely it is that he will develop a lifetime dependence on nicotine (Kandel and Chen 2000; Chassin et al. 1990). Several studies in rats have shown that adolescence is a critical period, but to

our knowledge, no studies in mice have been conducted. In addition to developing these models in another species, we sought to examine the effects of dose, exposure duration, and phase of adolescence in models of nicotine reward and withdrawal.

Using the CPP model as an indication of nicotine reward, we found that male adolescent mice exhibited enhanced rewarding effects in a dose- and duration-dependent manner. That is to say, a repeated adolescent nicotine exposure pattern (7 days) and a higher dose of nicotine (0.5 mg/kg) resulted in elevated reward levels in the CPP model when mice were tested in adulthood. Moreover, this result was unique to mice exposed during early adolescence (PND 22-28) and was not seen when mice were exposed to nicotine during late adolescence (PND 50-56) or adulthood (PND 71-77).

These same parameters were also used to assess adolescent nicotine exposure on withdrawal in adulthood. Similar to the reward model, we observed that early adolescent nicotine exposure caused a long-lasting attenuation of both somatic and affective withdrawal signs in adulthood. In contrast to our reward studies, intensity of withdrawal signs decreased in adulthood after both short- (3 day) and long-term (7 day) nicotine exposure. In addition, both high (48 mg/kg/day) and low (24 mg/kg/day) doses of nicotine produced this reduction. Late adolescent and adulthood mice tested in this paradigm did not show diminished withdrawal signs when evaluated in adulthood again supporting the hypothesis that early adolescence is a particularly distinctive period for the development of nicotine dependence.

Taken together, these studies confirm that early adolescent nicotine exposure results in long-lasting alterations in the behavioral response to nicotine. The rewarding

effects of nicotine are elevated in a dose- and duration-dependent manner, while nicotine withdrawal signs are attenuated independently of these factors. Our findings suggest that children and teenagers who begin smoking cigarettes at a young age are likely to have long-term consequences as a result of this behavior. Moreover, certain aspects of dependence can be observed after short exposure periods and exposure to low doses of nicotine. Indeed, in the clinical setting, studies indicate that dependence can be seen after smoking only a few cigarettes (DiFranza et al. 2002, 2007). Correlating this to a rodent model, significant upregulation of nAChRs was found after exposure to low doses of nicotine corresponding to a net consumption of just two cigarettes a day (after correction for species differences) (Lichtensteiger et al. 1988; Trauth et al. 2000). In summary, both animal models and human studies suggest that early age experimentation with cigarette smoking could have important implications on future nicotine dependence.

These findings also raise the question of how second-hand smoking effects in adolescence may affect levels of nicotine dependence. We have shown that relatively short periods of nicotine exposure and at low levels may cause alterations in important regulatory systems. Children who have parents or friends who smoke may be exposed to levels of nicotine which could detrimentally effect the development of neurological systems. These changes are likely to affect the reinforcing and aversive properties of nicotine and other drugs of abuse and may lead to increased vulnerability in these areas. In order to gain a more complete understanding of these possible consequences, studies

examining the effects of passive exposure to nicotine on later susceptibility to nicotine dependence should be conducted.

F. Exposure to nicotine during early adolescence has persistent effects on cocaine-induced behavior in adulthood.

Nicotine is one of the first and most commonly abused drugs in adolescence and is known to be a strong predictor of subsequent alcohol and other drug abuse (Kandel et al. 1992). Indeed, our data demonstrate the effects of adolescent nicotine exposure on cocaine-induced behavioral responses. We show that early adolescent nicotine exposure can enhance the rewarding effects of cocaine, cocaine-induced hyperactivity, and behavioral sensitization to cocaine. In both our CPP model and our acute locomotor studies, data showed that a repeated exposure to the higher dose of nicotine (0.5 mg/kg) was able to alter cocaine-induced responding. In contrast, neither acute exposure nor a low dose of nicotine (0.1 mg/kg) was able to elicit this effect. These findings have important implications for cross-sensitivity between nicotine and cocaine. It is likely that the mechanisms behind dependence to these two drugs share some commonalities.

Although drugs of abuse target several brain areas, enhanced dopamine transmission from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) is a key element in the reward (Koob and Le Moal 1997; Dani 2003). It is known that adolescent nicotine exposure has long-lasting effects on the development of various pharmacological systems and it is likely that the dopaminergic system is one which is greatly affected. Since nicotine and cocaine are both known to effect levels of

dopamine in the brain, this pathway is a likely candidate for the observed cross-sensitization. Although our data in Chapter 5 did not find significant differences between adults and adolescents in nicotine-induced dopamine release, it was not done after repeated exposure to nicotine. Therefore, it is still possible that dopamine plays a role in these changes. Furthermore, our technique used synaptosomes which are a crude preparation that does not preserve the intact neuronal connections between cells. It is possible that more precise techniques such as microdialysis would be more useful in this regard.

Other receptors may also be involved in our behavioral observations. Indeed, glutamatergic receptors are known to be involved in nicotinic effects as well. Adriani et al. (2004) demonstrated that adolescent, but not adult, nicotine exposure down-regulated mGluR2/3 subunits in the hippocampus and striatum. This same study also showed changes in NMDA NR2A/B subunits regardless of the time of exposure suggesting the involvement of NMDA receptors in certain aspects of nicotine dependence. These findings imply that other receptors may also be involved and should be further examined.

G. Conclusions and Implications

In summary, the research in this dissertation contributes to the further understanding of several components adolescent nicotine dependence. We are the first group to undertake a comprehensive study of nicotine reward and withdrawal investigating both age- and sex-related effects. In addition, we have established the role for a variety of factors such as dose, sex, and duration of exposure period. We have

found that adults and adolescents differ in their response to nicotine in both reward and withdrawal models. In addition, we have shown that males and females display opposing trends in these models. These data implicate the need for specifically targeted smoking cessation therapies in order to effectively reduce the number of unsuccessful quit attempts.

Our research has also shown that there in an increase in nAChR function in the adolescent rodent upon nicotine stimulation. This increase is function is not attributable to an increase in the basal number of receptors, but may involve other receptor regulation mechanisms such as desensitization or upregulation. Moreover, adolescent nicotine exposure also significantly enhances nAChR in adulthood, even after a drug-free period.

Finally, we have demonstrated in a mouse model that relatively low levels and short exposure to nicotine during adolescence has long-lasting effects on both nicotine and cocaine dependence in adulthood. Specifically, early adolescence represents a particularly unique period of development which is susceptible to these effects, while middle and late adolescent ages do not appear as critical. Our data show that adolescent nicotine exposure causes enhancement in both nicotine and cocaine-induced reward. Furthermore, these effects are dependent on the dose and duration of exposure. In contrast, adolescent nicotine exposure causes attenuation of nicotine withdrawal independent of treatment duration and dose. Nicotine treatment in early adolescence also elevated cocaine-induced hyperactivity and locomotor sensitization further

indicating its ability to induce neurochemical changes which alter susceptibility to drug dependence well in adulthood.

H. Future Studies

Studies indicate that nicotine, like cocaine, activates the mesocorticolimbic dopamine system suggesting that these two drugs of abuse share similar neurological mechanisms. Indeed our research shows that nicotine exposure is able to enhance the several effects of cocaine later in life. This data is novel and intriguing and has substantial implications in regards to adolescent smoking.

In our studies, relatively low levels of nicotine and short patterns of exposure were utilized demonstrating that even experimentation with cigarette smoking could have significant consequences. A study by Damaj et al. (2007) has shown that 60 min following a 2.5 mg/kg administration (s.c.) of nicotine the plasma level was approximately 40 ng/ml. Our study used a dose of nicotine five times lower than that in the previous study (0.5 mg/kg). Since the dose and plasma nicotine levels are linearly correlated (Lichtensteiger et al. 1988), this would indicate a plasma nicotine level of approximately 8 ng/ml in our experiments after 60 min. In addition, the maximal plasma level of nicotine (C_{max}) was equal to 314 ± 170 ng/ml in the study by Damaj et al. (2007). We would therefore expect a maximum plasma nicotine level in our study to be approximately 63 ng/ml. Our studies exposed animals to nicotine levels that are three times lower than the plasma nicotine levels reported for a typical smoker (25 ng/ml (Trauth et al. 2000)). Taken together, our findings show that short exposure to relatively low levels of cigarette smoke is likely to have detrimental and long-lasting

effects on drug dependence. Though this is the first study to examine these factors in the mouse model, our findings are in agreement with a study in adolescent rats which also found that the biological mechanisms underlying nicotine dependence can be activated by nicotine exposure comparable to that of an occasional smoker (Abreu-Villaca et al. 2003).

The mechanisms which underlie this "cross-sensitization" are still being elucidated. However, several future studies would be useful in determining these pathways. For example, nicotine may be altering dopamine receptor number or function or the level of dopamine transporters; therefore studies which measure DA receptor function and binding of DA ligands as well as DAT binding should be conducted. Specifically, D_1 and D_2 ligands are of particular interest. Functional assays such as GTPγS autoradiography would be a useful *in vitro* strategy in this regard. Moreover, studies have shown that there is an age-dependent development of dopaminergic receptors in that levels peak in adolescence before declining to adult quantities (Brenhouse et al. 2008). These changes in receptor quantity correlate with cocaine-induced responding in models such as conditioned place preference. The use of DA receptor agonists and antagonists may also contribute to further understanding. For example, agonists have been shown to enhance the rewarding effects of cocaine in juveniles which previously did not show cocaine preference (Brenhouse et al. 2008). The use of DA ligands may prove useful in smoking cessation treatment strategies and should be further explored.

Based on our findings, future studies should focus on a repeated exposure pattern during the early adolescent period. Our data showed that acute exposure was not sufficient to induced persistent behavioral alterations. It is likely that this exposure pattern is not able to activate important downstream effectors which contribute to these alterations. Recruitment of proteins involved in synaptic plasticity or gene expression are most likely required to induce such results. In summary, we are the first to see that after relatively short exposure periods of low doses of nicotine, there are long-term changes in the behavioral responses to cocaine. One particular challenge in explaining these findings is identifying fairly stable drug-induced changes which correlate to these behaviors. These types of alterations would add to the comprehension of our results.

Several factors may be contributing to our observations and should be further explored. Differences in memory storage, synaptic plasticity, and gene transcription and expression should all be considered. We have shown that there is increased nAChR function in both naïve adolescent synaptosomes and in adult synaptosomes following adolescent nicotine exposure. This increase in receptor function may translate into downstream consequences which may be more directly involved in the plasticity effects. In addition to changes in the dopaminergic system which have been previously discussed, it is important that other alternatives are considered.

Certainly, long-term effects may be mediated by drug-induced changes in gene expression. Given that the adolescent period is known to be a highly malleable phase of development in which there is an elevated level of synaptic remodeling (Rakic et al. 1994), it is probable that exposure to drugs of abuse is able to transform circuitry in the

brain and induce transcription factors which cause further long-term effects. Two transcription factors that have been strongly implicated in the addictive properties of drugs of abuse are CREB (cAMP response element binding protein) and ΔFosB.

Nicotine has been shown to induce the transcription factor ΔFosB, a member of the Fos family of transcription factors (Pich et al. 1997). ΔFosB is a good candidate for causing long-term plasticity effects in that it is rapidly induced, but is also very stable due to its long half-life (Chen et al. 1997). Studies with transgenic mice have allowed researchers to investigate the role of this transcription factor in the behavioral plasticity to drugs of abuse. In particular, a study by Kelz et al. (1999) found that mice overexpressing ΔFosB showed enhanced sensitivity to both acute locomotor effects and rewarding effects of cocaine. Indeed, an upregulation of ΔFosB due to adolescent nicotine exposure would explain our results which also demonstrated enhanced responding to cocaine's acute and rewarding effects.

In addition to research showing that nicotine is able to induce ΔFosB, there is also other evidence which may link this transcription factor with our data. ΔFosB is likely to act at other targets which play a role in nicotine and cocaine addiction. For example, the study by Kelz et al. (1999) also indicates that the GluR2 subunit of the AMPA receptor is a target of ΔFosB. Furthermore, they show that GluR2 expression is increased in the NAc following overexpression of ΔFosB. This study goes on to eloquently show that rewarding effects of cocaine are enhanced due to overexpression of the GluR2 subunit which gives another possible mechanism that would explain the data presented in Chapter 7. Since induction of ΔFosB is long-lived, but not

permanent, the upregulation of receptor subunits such as GluR2 in the AMPA receptor help to better explain why adolescent drug exposure may have effects of further drug abuse well into adulthood.

Other possible transcriptional mechanisms which should be considered when explaining our results is the induction of CREB by nicotine. Several studies have demonstrated a correlation between nicotine administration and CREB. In particular, Walters et al. (2005) have shown that activation of CREB is necessary for nicotine reward in adult mice as measured by conditioned place preference testing. Furthermore, chronic nicotine administration in mice results in decreased CREB phosphorylation in the NAc but increased CREB phosphorylation in the prefrontal cortex, while nicotine withdrawal increases CREB phosphorylation in the VTA (Brunzell et al. 2003). In contrast, another study shows that withdrawal from chronic nicotine in rats decreases CREB, phosphorylated CREB, and CRE-DNA binding in the cortex and amygdala (Pandey et al. 2001). While the precise role of CREB is not yet determined, it is clear that it is involved in both nicotine reward and withdrawal. Induction of the transcription factor CREB has been linked to an increase in the expression of tyrosine hydroxylase (Piech-Dumas and Tank 1999), an enzyme which is critically involved in the formation of dopamine. Even though the induction of CREB is relatively short-lived as compared to that of ΔFosB, it may still play a role in long-term plasticity changes via the enhancement of dopamine in the mesolimbic reward pathway.

In summary, it is probable that transcriptional mechanisms such as the ones described above are involved in the long-term plasticity effects of adolescent nicotine

exposure. It will be worthwhile to explore these mechanisms in greater detail in the future. Studies involving transgenic mice (both CREB and ΔFosB) as well as an investigation of pCREB and ΔFosB markers through molecular techniques such as western blots could add to the understanding of the behavioral results which are described in our research.

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VITA

Dena Heath Kota was born on February 17, 1982 in Norfolk, Virginia. Dena graduated from Hampton Roads Academy in May 1999 earning both the John Orders Senior Thesis Award and the Hampton Roads Academy Navigator Award; the highest award given to a graduating senior. She attended Mary Washington College in Fredericksburg, Virginia and competed on the NCAA Women's Intercollegiate tennis team earning all conference awards her junior and senior year. Dena obtained her Bachelor of Science degree in Biology in May 2003. Dena came to Virginia Commonwealth University in August 2004 and joined the Department of Pharmacology and Toxicology. She entered the lab of Dr. M. Imad Damaj in May 2005 and began her research on adolescent nicotine dependence.

In addition to her graduate work, Dena has presented numerous abstracts at conferences including annual meetings of the Society for Neuroscience and the Society for Research on Nicotine and Tobacco. She also served on the Pharmacology and Toxicology Student Organization from 2004-2007 and was an active member of VCU's Medical Campus Honor Council from 2004-2007, serving on the executive board from 2006-2007.

Manuscripts

•**Kota D**, Martin BR, Robinson SE, Damaj MI (2007) Nicotine dependence and reward differ between adult and adolescent mice. J Pharmacol Exp Ther 322(1):399-407.
•**Kota D**, Martin BR, Damaj MI (2007) Age-dependent differences in nicotine reward and withdrawal in female mice. Psychopharmacology (March 13, 2008 e-publication)

•**Kota D**, Martin BR, Damaj MI Nicotine exposure in early adolescence produces persistent behavioral effects in nicotine reward and withdrawal models in mice. (in preparation, JPET)
•**Kota D**, Martin BR, Damaj MI Adolescent nicotine exposure causes long-lasting changes in cocaine dependence (in preparation, JPET)
•**Kota D**, Martin BR, Damaj MI Early adolescence in a unique period for enhanced sensitivity to nicotine reward in mice: the role of acquisition and extinction (in preparation, Eur J of Pharmacol)

Abstracts
Kota D, Martin, BR, Damaj MI. Nicotine effects and dependence in male and female adolescent mice. *Virginia Youth Tobacco Project Annual Meeting*, March 2006

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